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Hcmv Car T Cells As A Novel Platform For Glioblastoma Cancer Immunotherapy

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Abstract

Glioblastoma multiforme (GBM) is the most common and deadliest primary brain tumor. Immunotherapeutic approaches using chimeric antigen receptor (CAR) T cells have shown limited efficacy against GBM due to heterogeneous target antigen expression. We hypothesize that human cytomegalovirus (HCMV) can serve as a therapeutic target for GBM. HCMV can be detected in up to 90% of GBM tumor samples but not the surrounding normal brain tissue. The role of HCMV as a tumor-promoting virus is poorly understood but its presence in the tumor presents a novel approach to developing a therapy for GBM by re-directing T cells to target HCMV. We detected the presence of HCMV in GBM tumor samples via IHC and confirmed expression of HCMV gene UL55/glycoprotein B (gB) in 45% of primary GBM tumors. A CAR was generated and optimized to recognize the HCMV surface antigen gB. In vitro testing of the anti-gB CAR revealed activity against the U87 glioma cell line stably transduced to express gB and CMV-infected human foreskin fibroblasts (HFF) cells. In vivo, gB CARs were able to treat established GBM tumors in a xenograft mouse model. In vitro co-cultures of gB CAR T cells against the human GBM explant, D270, demonstrated tumor recognition and anti-tumor function against primary GBM. gB CAR T cells were able to control D270 tumor growth in vivo despite undetectable levels of antigen expression. Mice displaying stable disease showed improved persistence of engrafted human T cells and tumor infiltration. These results suggest that CAR T cells may be effective in recognizing extremely low abundance antigens, and taken together, the results of this study show the feasibility of using gB CAR T cells as a platform to target HCMV in GBM tumors to treat patients with GBM. Ultimately, the goal of this study is translate these findings into clinical trials.

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HCMV CAR T CELLS AS A NOVEL PLATFORM FOR GLIOBLASTOMA CANCER IMMUNOTHERAPY

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Glioblastoma multiforme (GBM) is the most common and deadliest primary brain tumor. Immunotherapeutic approaches using chimeric antigen receptor (CAR) T cells have shown limited efficacy against GBM due to heterogeneous target antigen expression. We hypothesize that human cytomegalovirus (HCMV) can serve as a therapeutic target for GBM. HCMV can be detected in up to 90% of GBM tumor samples but not the surrounding normal brain tissue. The role of HCMV as a tumor-promoting virus is poorly understood but its presence in the tumor presents a novel approach to developing a therapy for GBM by re-directing T cells to target HCMV. We detected the presence of HCMV in GBM tumor samples via IHC and confirmed expression of HCMV gene *UL55*/glycoprotein B (gB) in 45% of primary GBM tumors. A CAR was generated and optimized to recognize the HCMV surface antigen gB. *In vitro* testing of the anti-gB CAR revealed activity against the U87 glioma cell line stably transduced to express gB and CMV-infected human foreskin fibroblasts (HFF) cells. *In vivo*, gB CARs were able to treat established GBM tumors in a xenograft mouse model. *In vitro* co-cultures of gB CAR T cells against the human GBM explant, D270, demonstrated tumor recognition and anti-tumor function against primary GBM. gB CAR T cells were able to control D270 tumor growth *in vivo* despite undetectable levels of antigen expression. Mice displaying stable disease showed improved persistence

of engrafted human T cells and tumor infiltration. These results suggest that CAR T cells may be effective in recognizing extremely low abundance antigens, and taken together, the results of this study show the feasibility of using gB CAR T cells as a platform to target HCMV in GBM tumors to treat patients with GBM. Ultimately, the goal of this study is translate these findings into clinical trials.

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INTRODUCTION

Chimeric Antigen Receptor T cells Re-directed to Cytomegalovirus as a Novel Target for Glioblastoma Multiforme Cancer Immunotherapy

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General Introduction

Glioblastoma:

Glioblastoma (GBM) is a highly lethal and intractable brain tumor with few therapeutic options. Recognized as a stage IV brain tumor by the World Health Organization (WHO), GBM is the most aggressive and most frequent form of brain cancer (Cheray et al., 2017; Wen & Kesari, 2008). GBM can develop in two forms. The first form of GBM arises spontaneously, typically in the elderly, and with no prior evidence of disease. Secondary GBMs arise from low-grade gliomas and eventually manifest into high-grade GBM. Unlike primary GBMs, the secondary form of GBM is less aggressive and presents with a better prognosis for the patient. This distinction is important as much of the therapeutic interventions used to treat patients with GBM are determined by the primary or secondary origin of the tumor (Lombardi & Assem, 2017). It is estimated that of the 14,000 cases of brain tumors diagnosed annually in the United States, GBM will account for 60-70% of new brain tumor cases (Wen & Kesari, 2008). Current standard of care for GBMs consist of maximal surgical resection of the tumor followed by concomitant chemotherapy and radiotherapy (Stupp et al., 2005). Even with the most recent additions to standard of care, GBM offers a grim prognosis with a median life expectancy of approximately 15 months after clinical intervention. Invariably, the tumor will recur in most patients, resulting in less than 10% of patients surviving up to 5 years (Stupp et al., 2005). The shortcoming of standard cancer treatment modalities is the non-specific nature by which these therapies target both malignant and non-malignant tissues (Phillips et al., 2006; Wen & Kesari, 2008).

No known risk factors have been associated with GBM tumors and the cell of origin for primary GBMs remains controversial. Unlike secondary GBM that stems from a known precursor cell, primary GBM has no known cell of origin (Alcantara Llaguno & Parada, 2016). Characterization of molecular pathways altered within individual GBM tumors has helped define treatment options and predict patient prognosis. Expression profiles based on common oncogenic pathways found in GBMs have distinguished GBM into four molecular subtypes: 1) Classical, 2) Mesenchymal, 3) Proneural, and 4) Neural (Cheray et al., 2017; Verhaak et al., 2010). Despite advances in basic understanding of the drivers of GBM, the disease remains intractable to clinical interventions and has a large unmet clinical need for improvements to the current standard of care (Stupp et al., 2005).

Standard cancer therapies and the development of immunotherapies:

Standard forms of cancer treatment, such as surgical resection, radiotherapy, and chemotherapy, are often imprecise in directly targeting the tumor; this results in a range of side effects that damage normal cells in addition to malignant cells (Phillips et al., 2006; Wen & Kesari, 2008). Improving the specificity of new class of drugs to precisely target the cancer cell will invariably help mitigate the side effects associated with standard forms of cancer treatment. In the last two decades, new classes of oncology drugs have emerged with the aim of specifically targeting the tumor. Small molecular inhibitors are pharmacological agents that interrupt the activity of proteins involved in driving

tumor growth. One of the first FDA approved targeted agents was Gleevec (Imatinib). Gleevec inhibits the aberrantly active fusion protein BCR-ABL that results from the genetic translocation of the Philadelphia chromosome. This therapy is capable of turning a life-threatening disease, chronic myeloid leukemia (CML), into a manageable one with a daily dose. However, Gleevec represents just one small success story in field of small molecular inhibitors (Deininger, Buchdunger, & Druker, 2005). More often than not, cancers develop resistance to targeted pathways over the course of treatment. Patients treated with Gleevec are prescribed second line drugs that combat CML when the disease develops resistance to the drug of the initial treatment (Rosti, Castagnetti, Gugliotta, & Baccarani, 2017). The use of targeted agents in oncology parallels the story of antibiotic use in infectious disease. Resistance mechanisms rapidly emerge, rendering new drugs infective against a dynamically evolving tumor.

On a similar trajectory, the development of cancer immunotherapies stem from basic understanding of the immune system. One of the earliest forms of experimental cancer immunotherapies was the use of high-dose human IL-2 at the National Cancer Institute (NCI) to boost immune responses against melanoma (Rosenberg et al., 1988). The use of exogenously administered molecules to manipulate a patient's immune system to attacking tumor cells forms that basis of many of the immunotherapies currently being developed. Monoclonal antibodies (mAb) targeting oncogenic proteins often amplified on the surface of tumor cells were some of the first clinically approved forms of cancer immunotherapy (Weiner, Surana, & Wang, 2010). Combining the potency of the immune system with a

refined focus on specific drivers of cancer growth resulted in new forms of drugs that offered greater precision than standard of forms of cancer treatment. Exemplifying this type of therapeutic is Trastuzumab (Herceptin), which is a mAb that selectively inhibits the function of oncogenic HER2 protein and is used in the treatment of breast cancer. Shifting the focus from targets on a cancer cell to targets found on T cells marked a turning point in mAb-based therapies. Checkpoint blockade immunotherapies (CBI) remove brakes that hinder the ability of the immune system to recognize malignant cells and mount an immune response to antigens found within a tumor (Wei, Duffy, & Allison, 2018). The ability to unleash the immune system upon a tumor with the administration of a CBI is a powerful tool that brought cancer immunotherapies to the forefront as a new treatment modality in oncology. Unlike traditional cancer therapies, immunotherapies do not directly target the tumor; instead, these drugs predominately work on the T cells that are poised to attack a tumor but may be limited by tumor-intrinsic immunosuppressive mechanisms.

The targeted therapies outlined above are off-the-shelf therapies that can be administered to any patient with tumors amenable to treatment, oncogene inhibition or immune targeting. Concomitant with the development of these immunotherapies has been the development of new experimental and highly personalized T cell-based immunotherapies. Adoptive cell transfer (ACT) of *ex vivo* expanded T cells is not off-the-shelf in its current form but instead offers the benefit of delivering a more dynamic “living drug” with pharmacokinetics that allows the therapy to work in the patient for years beyond a single dose (Kalos & June, 2013).

The Immune system and Cancer:

The development of ACT therapies stems from observations that the immune system can have anti-tumor activity. This relationship is complicated by the double-edged roles the immune system can play in the development of a tumor. On one hand, tumors are able to subvert immune responses by coopting immunosuppressive cells and mechanisms the immune system utilizes to prevent autoimmunity. On the other hand, the immune system can readily recognize tumors that have deviated from normal tissues as a foreign threat. The evolution of a tumor and its relationship to the immune system is reflected in the theory of cancer immunoediting (Mittal, Gubin, Schreiber, & Smyth, 2014). The growth of a nascent tumor can be broken down into three phases. First, in the elimination phase, the most immunogenic tumor cells are selectively deleted from the tumor mass by anti-tumor T cells. This process can occur over years, eventually establishing an equilibrium phase between the anti-tumor T cells and the immunosuppressive cells or molecules that the tumor uses to shutdown anti-tumor immune responses. Ultimately, this immunological détente is broken, resulting in the final phase of tumor escape. The end result of this process is a tumor mass virtually invisible to the immune system due to loss tumor of immunogenicity and/or the development of an immunosuppressive tumor microenvironment (Mittal et al., 2014). Understanding the relationship between a developing tumor and the immune system offers pathways that can be targeted as a mode of therapeutic

intervention. Cancer immunotherapies exploit the natural ability of T cells to recognize tumor cells and help tip the balance in favor of an anti-tumor response.

Adoptive T cell therapies for cancer:

The clinical use of cell therapies to treat human cancer is relatively new. The lab of Steven Rosenberg pioneered the use of tumor infiltrating lymphocytes (TIL) therapy. TIL are T cells that have trafficked to the tumor due to their ability to respond to an antigen presented by the tumor. These T cells are harvested from resected tumors and expanded *ex vivo* for ACT therapy. The NCI group has improved upon the ACT platform by incorporating a lymphodepletion regimen prior to TIL transfer in order to enhance engraftment of the newly introduced T cells (Rosenberg & Restifo, 2015). TIL therapies do not depend on identifying T cell specificities, instead by virtue of tumor residency all TILs were initially regarded as tumor reactive. TIL therapies rely on the existence of tumor-infiltrative and reactive T cells that can be expanded *ex vivo*. Melanoma is a particularly good model for TIL therapy, as these type of tumors are considered immunologically “hot” due to the high prevalence of neo-antigens that T cells can recognize (Schumacher & Schreiber, 2015). However, unlike melanoma, most tumors do not exhibit high mutational loads and may not contain pools of TILs for therapeutic intervention (Alexandrov et al., 2013). This limitation sparked a shift from expanding naturally occurring T cells found within a tumor to re-directing polyclonal T cells from the periphery to recognize tumor antigen via gene-transfer.

T cells could be re-directed to recognize tumor antigens via gene-transfer of an isolated TCR of known specificity (Dembic et al., 1986). Though this method, rare T cell clones found in a polyclonal TIL population can be reproduced on a larger scale as immunodominant effectors via TCR transduction of polyclonal peripheral blood T cells. This was demonstrated in a clinical trial using T cells re-directed to the melanoma antigen MART-1 (Johnson et al., 2009; Morgan et al., 2006). Other groups have followed suit by re-directing T cells to NY-ESO-1 in synovial sarcoma, leading to clinical objective responses in half of the patient cohort (Robbins et al., 2011). Using TCR gene-transfer to re-direct T cell specificity does have its limitations. TCRs require an exact match between a patient's human leukocyte antigen (HLA) and the TCR alpha (α) and beta (β) chains. If a patient does not share the same HLA-allele that the TCR recognizes, this therapy would not benefit the patient. Newer technologies bypass this limitation by re-directing T cells with gene transfer of synthetic molecules, CARs, which are designed in an HLA-independent manner.

Chimeric Antigen Receptor Design:

Unlike TCRs, CARs are synthetic gene constructs that incorporate domains from different molecules into one elegant and modular design (Kalos & June, 2013). The design of a CAR can be roughly broken down into three domains. The first is the recognition motif that allows detection of the surface antigen. Second is the hinge or spacer region that affects that ability of a T cell to recognize their cognate antigen. The last domain is the signaling domain that modulates and

allows for signal transduction upon ligation of the target antigen. The modular design of CAR molecules allows for rapid development of new CARs with novel specificities, each of which must be tested empirically in order to identify the lead construct. Because CAR T cells recognize antigen in a different fashion from TCRs, the biophysical interaction between a CAR T cell and target cell have also been altered (reviewed in Sharma and Kranz (2016)). Therefore, special considerations must be paid to the structure and expression of the target antigen and how these factors can influence CAR T cell effectiveness against a tumor cell. The remainder of this section will expand on the contribution of the three domains of a CAR molecule and how they can be used to guide CAR design (Kalos & June, 2013; Sadelain, Brentjens, & Riviere, 2013).

The recognition domain of a CAR construct confers a new specificity to a re-directed T cell. The ease of selecting a new specificity for CAR T cells is only limited by the expression of a target antigen on the surface of a cell. The most commonly used form of a recognition domain is based on a single chain variable fragment (scFv). ScFvs incorporate the variable regions of a monoclonal antibody and their specificity onto the signaling apparatus of the artificial T cell receptor. Moreover, the variable heavy and light chains can be swapped in orientation, which may change the sensitivity of the CAR to the target antigen (Burns et al., 2010). The recognition domain of a CAR can take on other forms like the natural ligand for a particular tumor antigen. This approach was utilized in the design of a “zetakines” targeting IL13RA2 in GBM, in which IL13 ligand was incorporated as the recognition domain of the CAR (Kahlon et al., 2004). The most recent approach

to the alteration of the CAR recognition domain comes in the form of chimeric autoantibody receptors (CAARs). CAARs fuse the target antigen of autoreactive B cells to selectively target those specific B cells for destruction. This approach was first published in a model of pemphigus vulgaris, where autoreactive B cells react to desmoglein 3 (Dsg3) (Ellebrecht et al., 2016). The specificity conferred by the recognition domain of CAR molecule warrants important consideration as it can directly contribute to off-target toxicities associated with CAR T cell therapies (reviewed in Antigen Selection and Safety considerations).

The hinge or spacer domain influences the manner in which the CAR T cell interacts with the target antigen. It is this domain that connects the recognition domain to the signaling domains and allows for fine-tuning of the sensitivity of the CAR to its cognate antigen (Ellebrecht et al., 2016; N. Watanabe et al., 2016; Ying et al., 2019). To illustrate the importance of the hinge domain, CARs were designed to recognize the tumor-associated antigen ROR1 based on two monoclonal antibodies targeting two different epitopes. The antibody epitopes were found to be either membrane proximal or on the apex of the ROR1 molecules, i.e. membrane distal. The high affinity anti-ROR1 R12 scFv mapped to a membrane proximal epitope on ROR1 and could only be recognized by CAR T cells bearing the long hinge variant of IgG4 and not the short, truncated hinge variants of IgG4. However, if a CAR construct incorporated the 2A2 scFv, which mapped to a membrane distal epitope, the short, truncated hinge was found to exhibit optimal T cell effector function *in vitro* (Hudecek et al., 2013). Moreover, the IgG4 hinge was found to have unexpected biological activity: the IgG4 hinge

interacted with murine Fc receptors in NSG mouse models (Hudecek et al., 2015). This interaction is not entirely unexpected as the IgG4 hinge contains an intact Fc portion capable of being engaged by Fc gamma receptors (FcγRs) receptors. This biological activity of the spacer domain on CAR T cell performance was initially reported in CAR constructs bearing IgG1 derived hinge domain (A. Hombach, Hombach, & Abken, 2010). Mutations in the CH2 domain of the IgG4 spacer improved the anti-tumor efficacy of the CAR T cells bearing the mutant IgG4 spacer by reducing activation induced cell death (AICD) (Hudecek et al., 2015; Jonnalagadda et al., 2015).

While the location of the epitope can influence the choice of spacer domain, there are no generalizable rules on the design of CAR for a particular target or epitope yet. The kinetic segregation model may help explain the relationship between the CAR's design and its ability to recognize target. This model outlines a mechanism by which a TCR signals or is inhibited by the spatial segregation of negative regulators of TCR signaling, such as the CD45 phosphatase. The large ectodomain of CD45 is excluded from the immunological synapse in instances where the TCR encounters its cognate antigen in the context of MHC (J. R. James & Vale, 2012). The spatial interaction between a T cell and its target remains constant for all TCR-MHC junctions, as CD45 is excluded from TCR-MHC immune synapses (Choudhuri, Wiseman, Brown, Gould, & van der Merwe, 2005). On the other hand, CAR T cells bearing synthetic receptors can exhibit variability in CD45 exclusion due to differences in CAR extracellular designs and require additional

optimization of the hinge domain for ideal CAR T cell signal transduction (Davis & van der Merwe, 2006; Huppa, Gleimer, Sumen, & Davis, 2003).

The selection of CAR signaling domains can be the most influential aspects of CAR T cell function. The first iteration of a CAR originated in the lab of Zelig Eschar (Eshhar, Waks, Gross, & Schindler, 1993). The basic design of the "T-body" fused an extracellular scFv to the CD3ζ signaling motif. The T-body design later became known as a first-generation CAR because it lacked additional co-stimulatory domains that augment the activation potential of CAR constructs. The CD3 ζ domain is sufficient to re-direct a T cell to perform cytolytic function but additional T cell functions require higher levels of stimulation that is provided by the incorporation of co-stimulatory domains. CARs that include additional signaling domains have led to clinical success and augmented CAR T cell function. Of note are the CD28 and 4-1BB (CD137) domains that have been evaluated extensively in both preclinical models and in the clinic. Studies comparing the activity of these two co-stimulatory domains in CAR T cells have revealed key differences. Biochemically, the incorporation of a 4-1BB domain alters a CAR T cell's metabolic programming, promoting fatty acid oxidation and enrichment of a central memory phenotype (Kawalekar et al., 2016). This is in contrast to CD28-based CAR T cells, which have glycolytic metabolism and an enriched effector phenotype. These differences may account for differences in the clinical persistence of CD19 re-directed CAR T cells, where 4-1BB-based CAR T cells persist longer than those based on CD28 (Maude et al., 2014; Park et al., 2018). These comparisons draw upon models targeting CD19 antigen, but does this superior activity apply to other

models? A GD2-specific CAR with a CD28 co-stimulatory domain was found to prematurely exhaust CAR T cells *in vitro* due to tonic signaling. Exchanging the CD28 co-stimulatory domain with 4-1BB diminished the exhaustion of GD2 CAR T cells (Long et al., 2015). These studies highlight the biochemical and clinical differences of CAR domains. Other co-stimulatory molecules have been evaluated in the context of CAR T cells, but it is unknown how they will compare clinically to the use of CD28 and 4-1BB.

Regardless of the approach undertaken to redirect a CAR T cell to a target antigen, optimization of the CAR construct may require several rounds of *in vitro* and *in vivo* testing in order to identify a lead construct with superior anti-tumor activity.

T cell biology:

Many of the considerations undertaken in the design of a CAR molecule draw upon the mechanistic understanding of T cell biology. T cell development, activation, and exhaustion inform some of limitations placed upon on a T cell and how they can be circumnavigated through the use of synthetic biology.

The ability of the immune system to recognize and clear foreign antigens while sparing endogenous self-antigens forms the first principles of T cell biology: the ability to respond to non-self. Selection of T cells occurs in the thymus where self-reactive T cells are selectively eliminated in two phases that evaluate the ability of a T cell to sense antigen (positive selection) and deletion of T cells that respond strongly to self-antigens (negative selection) (Klein, Hinterberger,

Wirnsberger, & Kyewski, 2009). This principle is important in cancer immunotherapy precisely because tumor cells derived from the same host as the immune system occupy a grey area in the self/non-self dichotomy. Chimeric antigen receptor (CAR) T cells bypass this natural limitation by re-directing post-thymic T cells to tumor-associated antigens (self-antigens) that are not exclusively expressed on the tumor. Thymic selection of T cells prevents development of autoimmunity via deletion of self-reactive T cells (central tolerance) but in turn may prevent robust immune recognition of a developing tumor that harbors mutated self-antigens (neo-antigens). Immunotherapies often blur the line between a normal immune response and autoimmunity to achieve therapeutic ends. This is best exemplified by CART19 T cell therapies that indiscriminately recognize CD19 antigen on both leukemic and normal B cells. CART19 T cell therapy can result in complete clearance of leukemic B cells but also leave the patient with B cell aplasia, a manageable form of autoimmunity (Grupp et al., 2013; Porter, Levine, Kalos, Bagg, & June, 2011).

The second basic principle of T cell biology focuses on post-thymic T cell activation. Naive T cells that have exited the thymus and have not been exposed to their cognate antigen must undergo a carefully controlled process that safeguards against inappropriate activation. Canonically, T cells must receive two signals in order to be fully activated; failure to acquire both signals would lead to an anergic T cell response (Smith-Garvin, Koretzky, & Jordan, 2009). All post-thymic T cells express a rearranged T cell receptor (TCR) that has been selected to respond to a foreign antigen. Engagement of a TCR to its cognate antigen in the

form a peptide-MHC complex provides “signal 1” of T cell activation. This signal is enhanced by the engagement of additional T cell molecules, known as co-stimulatory receptors, with ligands expressed on activated antigen presenting cells, an event that provides “signal 2”. Co-stimulatory receptors, such as CD28, 4-1BB, ICOS, and OX40, augment the strength of signal one and allow for full T cell activation (Chen & Flies, 2013). It is at this stage that CD8+ cytotoxic T cells or CD4+ helper T cells are able to perform their effector functions after encountering their cognate antigen once again on foreign, malignant, or infected cells. Basic understanding of the molecular mechanism of T cell activation has improved the function of CAR molecules and their performance in the clinic. CAR molecules bearing only a CD3ζ chain intracellular motif form the basis of “first generation CARs.” These CAR constructs are immunologically equivalent to receiving only “signal 1” via TCR engagement of cognate antigen. The addition of the intracellular signaling domain from a co-stimulatory molecule in the design of second generation CAR constructs has improved the clinical performance of CD19 CAR T cells (Kalos & June, 2013; Milone et al., 2009; Sadelain et al., 2013).

As the basic understanding of T cell biology continues to shed light into new targets for immunotherapy, the focus has now shifted to understanding how to prevent effector T cells from succumbing to T cell exhaustion. The progressive loss of T cell effector function due to chronic antigen stimulation renders anti-tumor T cell responses ineffective. Exhausted T cells can also be identified by their expression of inhibitory receptors, such as cytotoxic T lymphocyte antigen 4 (CTLA-4) or programed cell death protein 1 (PD-1) (Wherry & Kurachi, 2015).

Blockade of these inhibitory receptors has been the focus of new CBIs that seek to ameliorate the effects of exhaustion by blocking these pathways (Wei et al., 2018). Blockade of CTLA-4 molecule may work by lowering the threshold of activation for T cells that may respond to a self-antigen (Fife & Bluestone, 2008), widening the window of T cells capable of responding to the tumor. PD-1 and PD-L1 blockade seeks to reinvigorate T cells that are experiencing exhaustion by blocking the signaling pathway that would maintain the exhausted phenotype (Wei et al., 2018). CBIs are powerful tools in oncology that can unleash dynamic anti-tumor responses with an off-the shelf application. However, not all patients may respond to CBI therapy. T cell exhaustion may be irreversible in some T cells that have been epigenetically reprogrammed to be unresponsive to CBI therapy (Pauken et al., 2016). Moreover, tumors that carry low mutational burden may not respond to CBI therapies. The positive correlation between a tumor's mutational burden and clinical responses been reported for both PD-1 and CTLA-4 (Rizvi et al., 2015; Snyder et al., 2014; Yarchoan, Hopkins, & Jaffee, 2017). Patients that do not respond to CBI therapies may benefit from other T cell therapies, such as the adoptive transfer of T cells enriched for a certain tumor target specificity.

Antigen Selection and Safety considerations:

As reviewed in the previous section, the immune system has mechanisms of safeguarding against the development of autoimmunity. Many antigens targeted by re-directed T cells are derived from self-antigens. For this reason, target antigen selection is arguably the most critical consideration in the design of a T cell therapy.

The expression and biology of the selected antigen can make the difference between a beneficial clinical outcome, autoimmunity, or even death of a patient. ACT therapies have a short but telling history of how antigen selection translates in the clinic. Broadly, tumor antigens can be categorized as a tumor-associated antigen (TAA) or a tumor-specific antigen (TSA). The type of target antigen, TAA pr TSA has implications for the clinical activity of re-directed T cells.

TAAs represent the risker group of tumor antigens on the basis that their expression is not confined exclusively to malignant tissue. Targeting a given TAA profile can be acceptable if expressed on non-essential cells or it can have lethal consequences if found on vital organs. For example, clinical trials using CAR T cells designed with the scFv derived from the monoclonal antibody trastuzumab (Herceptin), which targets the TAA HER2, led to the death of a patient due to acute “on-target, off-tumor” toxicities in the lungs (Morgan et al., 2010). It bears mentioning that while Herceptin is FDA approved with known toxicities, the same antibody had greater and unexpected toxicities when expressed in CAR T cells. MAGE-A3 is a TAA that belongs a class of antigens known as cancer-testis antigens (CTAs). Given the limited expression profile of CTAs, they were thought to be safer than other TAAs, but T cells engineered to target MAGE-A3 have led to severe adverse events. Two clinical trials in which T cells were re-directed with TCRs targeting MAGE-A3 had unforeseen “off-target, off-tumor” toxicities due to cross-reactivity. In one trial, re-directed T cells recognized other MAGE-A family proteins in brain tissue, which led to the fatality of patients (Morgan et al., 2013). In the other trial, which using affinity-enhanced TCRs to target MAGE-A3, a patient

developed severe cardio-toxicities when the re-directed T cells cross-reacted with the protein titan (Linette et al., 2013). While these complications were not predicted before administration of the T cell therapy, there may be ways to mitigate the toxicities of redirected T cells responses utilizing new technologies for antigen selection.

The clinical trials outlined above represent some of the more severe outcomes associated with ACT of re-directed T cells; other trials have found success in targeting TAAs with tolerable toxicities. T cells redirected to MART-1 using human or mouse TCRs found tumor regression in patients with melanoma. However, patients enrolled in the clinical trial also exhibited toxicities in tissue with normal melanocytes (Johnson et al., 2009). Clinical trials targeting the CTA NY-ESO-1 had objective responses (OR) in 4 out of 6 patients with synovial cell sarcoma and 5 out of 11 patients with melanoma (Robbins et al., 2011). The most successful application of targeting a TAA comes from CAR T cells re-directed to the B cell antigen CD19 (Grupp et al., 2013; Porter et al., 2011). A single infusion of CD19 CAR T cells led to complete remission in 90% of patients with relapsed or refractory acute lymphoblastic leukemia (ALL). The most common severe adverse event (SAE) in early clinical trials of CD19 CAR T cells was cytokine release syndrome, which is mitigated by anti-IL-6R or anti-IL-6 monoclonal antibody therapy (Grupp et al., 2013). Despite these astonishing clinical outcomes, the therapy is not without its trade offs, as all responding patients experience B cell aplasia, which can be remedied with antibody replacement therapy (Maude et al., 2014). Hematological malignancies are proof-of-concept that CAR T cell treatment

can lead to lasting clinical results; however, the lion's share of human tumor burden lies in solid tumors (Siegel, Miller, & Jemal, 2019).

Solid tumors present a special problem in that tumor antigen expression can overlap with vital organs and thus needs to be approached with extra caution. One report using an affinity-tuned scFv to either EGFR or HER2 antigen desensitized CAR T cells to normal cells that express these TAAs and helped to improve selectively for tumors that overexpress these TAAs (Liu et al., 2015). Another study found that using a 4-1BB co-stimulatory domain on a PSCA CAR, instead of a CD28 domain, mitigated off-target toxicities in a xenograft model (Priceman et al., 2018). These design considerations could be implemented to improve the safety profile of CAR T cells re-directed towards TAAs but the safer alternative is re-directing T cells to target TSAs.

TSAs are expressed exclusively on tumor cells and can be immunogenic because they mark a clear destinction from normal, unmutated self-antigens. TSAs that are shared among different patient tumors are rare and desirable targets. A prime example is the TSA EGFR variant III (EGFRvIII). EGFRvIII is a splice variant of the receptor tyrosine kinase EGFR that is often found amplified in many malignancies. Genetically, the splice variant joins exons 1 and 8 in a novel molecule that results in constitutive activation of EGFRvIII. Consequently, the fusion between exons 1 and 8 of EGFR also forms an immunogenic epitope that can be detected by the immune system (Gan, Cvrljevic, & Johns, 2013; Sampson et al., 2014). On the basis of this finding, CAR T cells and vaccine approaches have been developed to target EGFRvIII (Johnson et al., 2015; Sampson et al.,

2009). A more encompassing but highly personalized approach to identifying TSA is to scan the tumor exome for potential neo-antigens. This approach was first reported in a case involving a patient with metastatic cholangiocarinoma. A CD4+ T cell clone reactive to a mutation in ERBB2IP was identified within the patient's tumor. Adoptive transfer of *ex vivo* expanded, ERBB2IP-specific reactive T cells mediated regression and tumor control (Tran et al., 2014). This approach was further advanced through the targeting of mutations in common oncogenes, as in the report where a T cell clone reactive to $KRAS^{G12D}$ was isolated and used to treat patients with $KRAS^{G12D}$-driven tumors (Tran et al., 2015; Tran et al., 2016). Taken together, the advent of technology that can pan for TSAs and the facile use of gene-transfer of TCRs or CARs in T cells will help accelerate the development of new ACT therapies that target TSAs.

While the dichotomy of TAAs and TSAs remains an important consideration in target selection, another class of TAAs blurs this distinction. Viruses and their gene products are highly immunogenic due to their foreign nature and are readily detected by the host immune system. The capacity of some viruses to transform an infected cell can present a platform by which T cells can be re-directed to kill tumor cells. *Ex vivo* expanded T cells specific to Epstein-Barr virus (EBV) were adoptively transferred to patients with various EBV-associated malignancies. Complete responses were observed in 4 out of 7 patients, demonstrating the feasibility of this cancer immunotherapy approach (Bollard et al., 2018).

Viruses and cancer:

Viruses play a special role in the history and study of cancer research. Through investigation of the cause of a transmissible form of sarcoma in chickens, the nature of oncogenes in humans was ultimately illuminated. The Rous sarcoma virus (RSV) was observed to reproducibly transform infected chicken cells *in vitro* and served as tool to understand the genetics of cancer (Bister, 2015). Hidden within the viral genome of RSV, lies the *vSRC* (viral Sarcoma) gene, which was found to be the genetic driver of transformation in infected chicken cells (Duesberg & Vogt, 1970; L. H. Wang, Duesberg, Kawai, & Hanafusa, 1976). This discovery, in itself, might seem trivial to a researcher outside the field of tumor virology; however, the true implications of this discovery only became apparent when a larger form of the *vSRC* gene was also found in the host cell genome (Stehelin, Varmus, Bishop, & Vogt, 1976). What was true for the chicken was also true of man, as the human genome also carried a SRC homologue. The genetic underpinnings of cellular transformation were not entirely foreign entities but rather endogenously found scattered throughout the human genome. Proto-oncogenes, as these genes were later named, are the genes that can drive cellular transformation when their function is altered via mutation (Vogt, 2012). Oncogenes also underscore an immunotherapy dilemma: if tumors can arise from spontaneous mutations in self-antigens, how can they be safely targeted with re-directed T cells? Categorically, mutated self-antigens occupy an immunological grey area that renders them difficult to target due to immunological tolerance as outlined in the previous section. In contrast, oncogenic viruses and their gene

products do not share this feature in that viral gene products are readily detected by the immune system as foreign antigens.

Given the history of viruses and cancer, it is not surprising that oncogenic viruses account for 15-20% of all human malignancies worldwide (Mesri, Feitelson, & Munger, 2014; Mittal et al., 2014). Oncoviruses, such as EBV, human papillomavirus (HPV), and Kaposi's sarcoma-associated herpesvirus (KHSV), have been directly linked to cell transformation via a myriad of viral gene products that inactivate host tumor suppressor genes or mimic the function of oncogenes (reviewed in (Krump & You, 2018). Viewed through the lens of cancer immunotherapy, targeting foreign antigens expressed by oncogenic viruses presents an attractive platform to treat virus-associated malignancies. GBM does not have an association with a causally linked oncogenic virus; however, reports have demonstrated that human cytomegalovirus (HCMV) resides within GBM tumor cells. HCMV has reportedly been found in many cancer types, including low- and high-grade gliomas, EBV-negative Hodgkin's disease, prostate and colorectal cancers (C. S. Cobbs et al., 2002; Harkins et al., 2002; G. Huang et al., 2002; Libard et al., 2014; Scheurer, Bondy, Aldape, Albrecht, & El-Zein, 2008). Unlike oncogenic viruses with a known mechanism of transformation, HCMV has not been linked to cancer transformation, instead it has been designated as an onco-modulatory virus (Dziurzynski et al., 2012). Onco-modulatory viruses may confer a tumorigenic phenotype unto an infected cell via the function of viral gene products. The high association of HCMV with GBM and reports of its presence in

other malignancies has captured the attention of clinicians seeking to target this virus for cancer treatment.

Natural History and the immune response to HCMV:

HCMV is a ubiquitous DNA virus with population infection rates that range from 50-80% based on different factors (Roddie & Peggs, 2017). Acute infection is often asymptomatic and results in a life-long chronic latent infection in people exposed to the virus. Fulminant HCMV reactivation develops in the context of immune suppression or in individuals with a compromised immune system. Of particular note are patients that have undergone an organ transplant or allogeneic stem cell transplant are at high risk to develop HCMV reactivation (Gandhi & Khanna, 2004). It is the window of immune reconstitution after HSCT that the virus can come out of dormancy from the reservoir cells. Typically, the main viral reservoir for HCMV is considered to the be CD34+ cells of the HPSC compartment (Sinclair & Sissons, 2006). It is in these cells that the virus can express a latent gene expression profile and avoid cellular immune responses. Monocytes derived from infected CD34+ progenitors can initiate lytic infection when these cells undergo differentiation in peripheral tissues (Sissons, Bain, & Wills, 2002). This shedding into the periphery is subverted and kept under control by a strong cellular immune response and is sufficient to control and prevent HCMV reactivation. It is estimated that approximately 10% of the memory CD4+ and CD8+ T cell compartment is devoted to responding to HCMV infection (Sylwester et al., 2005). The importance of a cellular immune response is also reflected in clinical studies

that performed adoptive transfer of HCMV-specific T cell clones to reconstitute anti-HCMV immunity in patients after a bone marrow transplantation (Riddell et al., 1992; Walter et al., 1995). However, if a cellular immune response is not available reactivation can lead to clinical complications. The high morbidity associated with HCMV has prompted the development of vaccines targeting immunodominant viral antigens. To date, no prophylactic vaccine has been approved for HCMV. This could be attributed, in part, to the highly adept ability of HCMV to evade and subvert the host immune response. HCMV produces a viral homolog of IL-10, which dampens immune responses and down regulates HLA presentation of viral peptides. Moreover, NK cells are evaded via a viral protein that mimics the function of HLA (Michaelis, Doerr, & Cinatl, 2009). Taken together, while the immune system is able to mount a robust immune response and suppress reactivation, but it is not sufficient to rid a host of the HCMV reservoir. Vaccinations based on HCMV antigens result in potent humoral immune responses and the viral antigen glycoprotein B (gB) has been identified as a vaccine candidate (Lilja & Mason, 2012). The humoral immune responses to gB are not effective at preventing infection and the robust response to the immunodominant epitope produces non-neutralizing antibodies, which outcompete neutralizing antibodies (Speckner, Glykofrydes, Ohlin, & Mach, 1999). The natural history of HCMV infections underscore why the virus is prevalent among humans and in addition why it is able to persist in a human host.

HCMV and GBM:

The association between HCMV and GBM was first reported in 2002. Charles Cobbs, a neurosurgeon at University of Alabama was interested in discovering the underlying cause of inflammation found in GBM tumors. The report demonstrated the presence of CMV antigens and nucleic acids in GBM tumors but not the surrounding normal brain tissue (C. S. Cobbs et al., 2002). Following the first report, a group at Duke University demonstrated the presence of the virus in up to 90% of GBM tumor samples collected at their medical center (Mitchell et al., 2008). Despite initial reports demonstrating positive associations between GBM and CMV, the reproducibility of these findings has been called into question as other groups have failed to detect HCMV in their GBM samples (Davis & van der Merwe, 2006; Garcia-Martinez et al., 2017; Priel, Wohl, Teperberg, Nass, & Cohen, 2015). As Cobbs argues in a response letter, the negative results ultimately falls upon the sensitivity and optimization of the methods used to detect the virus (Cobbs, 2014).

Given the propensity of HCMV to be confined within GBM tumor and not the surrounding normal brain tissue, clinicians have set out to leverage the tumor-specific localization as a platform for GBM immunotherapy. Early evidence of the ability to detect CMV in GBM tumors came from a dendritic cell vaccine pulsed with autologous GBM tumor lysate. After one vaccination, a dramatic increase of T cells recognizing the immunodominant epitope of CMV, pp65, was observed in one patient (Prins, Cloughesy, & Liau, 2008). Other groups set out to determine if patient-derived HCMV-specific T cells expanded *ex vivo* can detect autologous

GBM tumors *in vitro*. The results of this study found that specific T cells were able to sense and kill virus-infected GBM cells (Nair et al., 2014). These reports demonstrate that T cells are sensitive enough to detect CMV antigens on tumor cells and are sufficient to trigger T cell killing of the infected tumor cell.

Building on these clinical studies is a series of clinical trials using dendritic cell vaccines pulsed with CMV antigen pp65 for GBM immunotherapy. Initial reports used dendritic cells pulsed with pp65 mRNA and tetanus toxoid adjuvant. The mechanism underlying a robust immune response to the tumor was dependent on CCL3 (Mitchell et al., 2015). Follow-up studies have used variants of dendritic cells pulsed with pp65 and high intensity chemotherapy with varying levels of success (Batich et al., 2017). One study combined both adoptive cell transfer of HCMV-specific T cells in conjunction with dendritic cells pulsed with pp65. The result was restoration of polyfunctionality of adoptively transferred T cell uncovering a potential new mechanism of dendritic cells to rejuvenate exhausted T cells (Reap et al., 2018). Collectively these clinical studies demonstrate the feasibility and potential benefit of targeting CMV in GBM.

Targeting of GBM using anti-CMV CAR:

Missing from the clinical studies outlined in the previous section is an immunotherapy approach using CAR T cells re-directed to CMV as a way to target GBM. Unlike TCR-mediated recognition of target via MHC, CAR T cells rely on surface expression of the target antigen to initiate killing. Given so, the viral

genome of CMV encodes a variety of surface proteins amenable to CAR recognition. The ideal CMV antigen would be 1) a surface-bound molecule, 2) highly conserved among clinical strains of CMV, and 3) abundantly expressed on the tumor cell. One HCMV antigen that meets these criteria is gB, which is an extracellular viral antigen involved in the process of membrane fusion between CMV envelope and host cells (Gardner & Tortorella, 2016). Given the role in viral dissemination and cell entry, gB was used as a vaccine antigen in clinical trials but has not resulted in protection from HCMV infection (reviewed in (Anderholm, Bierle, & Schleiss, 2016). Despite these set backs, the humoral immune response induced in response to gB has yielded valuable information about epitopes found on gB that can guide CAR design (Ohlin & Soderberg-Naucler, 2015). Humoral immune responses to HCMV often fail to neutralize the virus due to competition for the immunodominant epitope of gB (Speckner et al., 1999). Moreover, neutralizing antibodies bound to gB can be internalized and incorporated into the viral envelope. Paradoxically, Fc receptor engagement and engulfment of a neutralizing antibody bound to gB on the HCMV envelope enhances the spread of HCMV into immune cells that the virus would not normally infect (Manley et al., 2011). Mechanisms of evading humoral immune responses directed against gB demonstrate the importance of maintaining a functional gB in the HCMV lifecycle (Gardner & Tortorella, 2016) Lastly, surface expression of gB has been reported in GBM (Mitchell et al., 2008; Ranganathan, Clark, Kuo, Salamat, & Kalejta, 2012). Investigators found that gB augments tumorigenicity of GBM via interaction of PDGFRα and gB (C. Cobbs et al., 2014). The association of PDGFRα with gB is

not surprising, given that the same group discovered that gB utilizes PDGFRα as an entry receptor into host cells (Soroceanu, Akhavan, & Cobbs, 2008). Taken together, the extracellular expression of HCMV gB on GBM tumors presents a target that is amenable to CAR recognition and has potential use for GBM immunotherapy.

We set out to evaluate the anti-tumor activity of CAR T cells re-directed to gB as a platform for GBM immunotherapy.

Chapter 2:

Detection of HCMV antigens within GBM tumor samples

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Introduction

HCMV was initially reported to be found in GBM in 2002 (C. S. Cobbs et al., 2002). Soon after the report was published, other labs reported not being able to replicate the initial reports (reviewed in (Rahman, Dastmalchi, Karachi, & Mitchell, 2019). Detection of HCMV in GBM tissue is fraught with methodological and sample collection issues (C. Cobbs, 2014). Sensitive detection of the low levels of HCMV requires optimized protocols and high quality specimens (C. S. Cobbs, Matlaf, & Harkins, 2014).

How HCMV contributes to GBM's tumorgenicity/tumorigenesis has been a topic of consideration. Of interest are the host factors found within tumor cells and how they can contribute to HCMV infectivity. GBM is a highly heterogenous tumor and can be defined by molecular alterations and deletions found within the tumor. The four subtypes that emerged from molecular characterization of GBM are the classical, proneural, mesenchymal, and neural (Verhaak et al., 2010). Amplification of EGFR and PDGFRα in certain GBMs may render these tumors more susceptible to HCMV infection. The degree of HCMV infection could hold prognostic value for patients with GBM. GBM tumors for immunohistochemistry (IHC) stained for HCMV IE1-72 were stratified into low- and high-grade infection groups. Patients with tumors displaying a low-grade infection had longer survival (Rahbar et al., 2013), suggesting that HCMV may contribute to the pathogenesis of GBM. In this study, we set out to confirm the presence of HCMV in GBM sample collected at the Hospital of the University of Pennsylvania (HUP). Additionally, we

aimed to investigate the role GBM subtypes may have on the expression of HCMV antigen gB.

Results

HCMV IE1-72 expression detected in primary GBM tumors

The presence of HCMV antigens has been reported in a high percentage of GBM cases (C. S. Cobbs et al., 2002; Mitchell et al., 2008; Scheurer et al., 2008). In order to verify this association, we performed IHC on nine primary GBM samples acquired at HUP. Sensitive IHC detection of HCMV IE1-72 protein was optimized to detect low levels of HCMV antigen expression typically found in GBM (C. S. Cobbs et al., 2002; Mitchell et al., 2008; Scheurer et al., 2008). Seven out of nine (77%) primary GBM cases showed positive staining for HCMV IE1-72. A high degree of heterogeneous HCMV IE1-72 antigen expression was found within the tumor and among the cases screened. No immunoreactivity was found in sections where the primary antibody was excluded (Fig. 3.1). Given the high prevalence of HCMV in GBM samples acquired at the Hospital of the University of Pennsylvania (HUP) and the reported expression of other HCMV antigens in GBM (Rahman et al., 2019), HCMV became attractive as a target platform to re-direct T cells to GBM.

CMV is preferentially found in the proneural subtype of GBM

We next sought to confirm expression of CMV gene *UL55* (glycoprotein B) in 40 primary GBM tumor samples collected at the Hospital of the University of Pennsylvania. The 40 GBM samples were designated into one of the four GBM

molecular phenotypes (Pal et al., 2014; Verhaak et al., 2010) and were screened for gB expression using a sensitive nested RT-PCR (C. S. Cobbs et al., 2014). Of the 40 samples, 18 tested positive for gB expression (45%) confirming similar findings by other groups (Mitchell et al., 2008; Ranganathan et al., 2012) (Fig 3.2). Interestingly, we observed preferential expression of gB within the proneural subtype (8/8). We hypothesize that the enrichment of expression of gB within the proneural subtype correlates with the ability of this gB to ligate PDGFRα and induce mitogenic signaling (C. Cobbs et al., 2014; Soroceanu et al., 2008). Taken together, the high incidence of CMV antigens present in GBM samples and the confirmation of gB expression in primary tumors could be used as a platform to re-direct T cells to GBM.

Materials and methods

Detection of HCMV antigens in primary GBM tumors

Both detection of IE1-72 antigen on primary GBM FFPE sections using IHC and the sensitive RT-PCR detection of *UL55* expression in cDNA generated from primary GBM followed the protocol outlined in C. S. Cobbs et al. (2014). PCR reactions were prepared in an AirClean 600 PCR workstation (AirClean Systems). Positive control reactions were prepared last and run on a separate PCR machine in a different room to avoid cross-contamination. Human adult normal cerebral brain cortex cDNA were commercially obtained (BioChain). Primers used for amplification were as follows:

gBF-external 5'- TCCAACACCCACAGTACCC-3';

gBR-external 5'-CGGAAACGATGGTGTAGTTCG-3';

gBF-internal 5'-CCGCCCGCCCCGCGCCCGCCGCGGCAGCACCTGGCT-3';

gBRinternal 5'- GTAAACCACATCACCCGTGGA-3'.

Microscopy

Microscopy was performed using a Leica DFC7000 system. Bright field images were taken in regions representative of IE1-72 staining grade. Automatic contrast was applied to images.

Discussion:

The central questions of this chapter are: can we detect the virus in samples derived at HUP and how do host factors influence HCMV infectivity of GBM? Sensitive detection of HCMV in GBM FFPE sections revealed a high degree on HCMV infection in GBM samples acquired at HUP. The staining intensity of IE1-72 varied among cases but HCMV can be detected with an optimized protocol. Given the high degree of optimization required to detect the HCMV, no protocol has been published for IHC detection of gB. It is important develop a baseline method of detecting HCMV by using IE1-72 staining before optimizing a gB staining protocol. This would reduce the chances of a false negative result by first detecting IE1-72 and then staining for gB. Due to HCMV expression kinetics during lytic infection, gB is expressed approximately two days after lytic infection, while IE1-72 is expressed at all stages of lytic infection (Radsak et al., 1996).

The contribution of host factors in facilitating HCMV infectivity in GBM warrants additional experiments. HCMV utilizes host factors for cell entry; of note are the RTKs EGFR and PDGFRα that are often amplified in certain GBMs (Gardner & Tortorella, 2016). The results reported here suggest that gB expression

might be highest in proneural subsets of GBM but does not preclude the presence of the virus in the other GBM subtypes. High levels of gB expression in proneural GBMs make gB CAR T cells good candidate for patients with proneural GBMs. However, one must consider the limitations of the technology used to subtype GBMs. Small clusters of cells might influence the subtype a certain GBM is designated. Moreover, subtyping presents a snapshot of the tumor sample at the time of collection. As it has become evident, GBMs undergoes tumor evolution from initial diagnosis and recurrence (Q. Wang et al., 2017). The morphing of GBM from one subtype to another in response to treatment has also been reported. Clonal variation within a GBM selects for pre-existing resistant clones to dominate the tumor mass. Proneural GBMs often morph into radiotherapy- and chemotherapy-resistant mesenchymal GBMs (Segerman et al., 2016). Thus, subtyping of GBM with regard to HCMV may not be as useful as initially thought. Given these results, the question of what subtype might be enriched for HCMV should be modified to consider the effect of treatment on HCMV status. Does HCMV status change from initial diagnosis and at reoccurrence? If GBM subtype transitions occur, do the associated changes in the tumor and TME favor HCMV infection of GBM or dissemination of HCMV into the tumor site? Mesenchymal GBMs found at reoccurrence are often found to be associated with an influx of macrophages, a known carrier of HCMV (Sinclair & Sissons, 2006; Q. Wang et al., 2017). HCMV's ability to infect different cell types within the TME brings up the question of which cells in the TME harbor the virus. This question has been partially answered by a report demonstrating that glioma cancer stem cells and glial cells

are enriched for the virus (Dziurzynski et al., 2011). Additional experiments determining the *in situ* localization of HCMV within specific cell types could illuminate potential targets for gB CARs within the TME.

Experiments to address some of the questions listed above would require a set of matched GBMs at initial diagnosis and again at reoccurrence. These matched samples can be IHC stained for IE1-72 and pp65 to determine if HCMV infection in GBM tumor tissue spreads or is reduced after treatment. Markers for glioma stem cells can be used in conjunction with HCMV IE1-72 to determine co-localization of virus and glioma cancer stem cells (gCSCs) or tumor-associated macrophages (TAMs). Additionally, a validation set of subtyped GBMs can confirm the incidental finding that proneural GBMs are enriched for gB expression using a nested PCR reaction for gB.

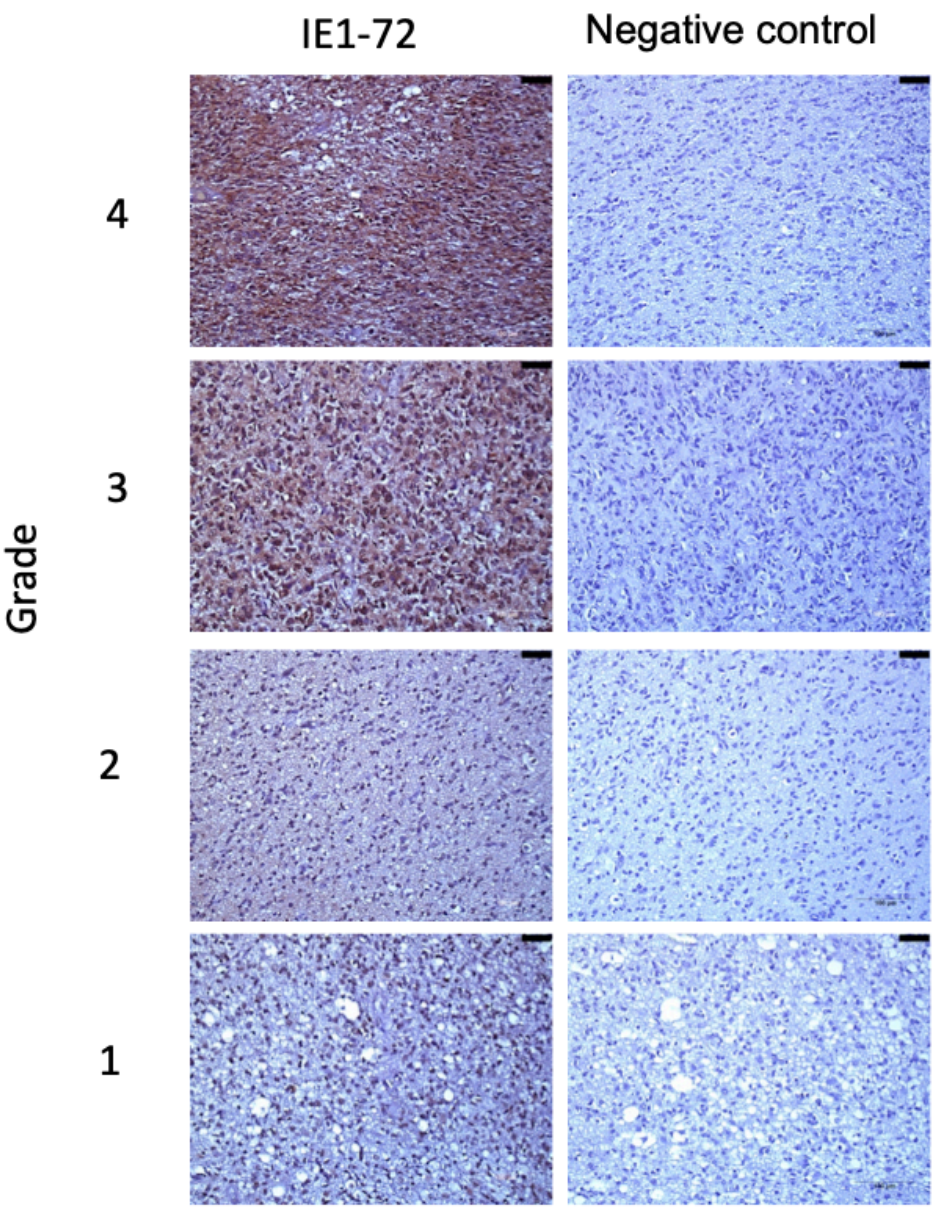

Figure 2.1. Immunohistochemistry (IHC) for CMV antigen IE1-72 on primary glioblastoma tumors. IHC was performed on 9 formalin fixed paraffin-embedded (FFPE) glioblastoma samples collected at the University of Pennsylvania hospital. 9 primary GBM samples were graded on a 0-4 scale for IE1-72 positivity, with grade 0 indicating IE1-72 negative, and grades 1-4 as positive with increasing staining intensity. Shown are representative images for each staining grade. Magnification 200x. Negative control sections consisted of no addition of the primary antibody for IHC.

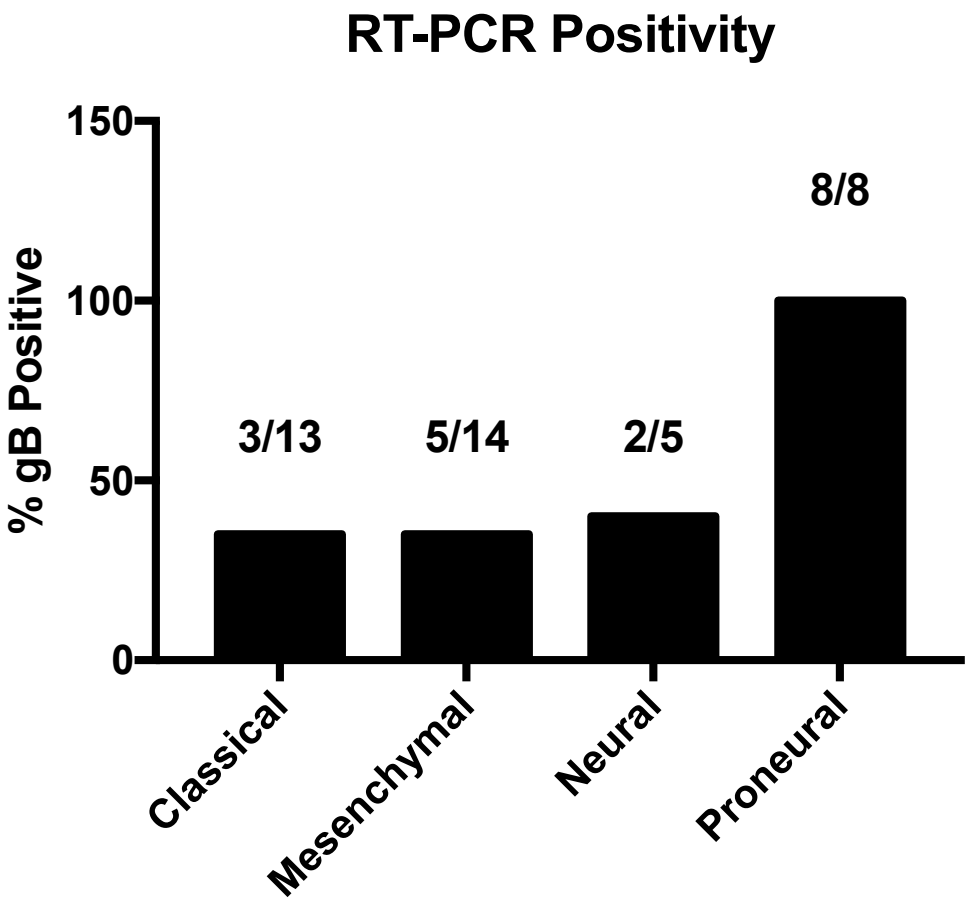

Figure 2.2. CMV antigen gB is expressed in primary GBM tumor tumors. Complimentary DNA (cDNA) from 40 primary GBM tumor samples were screened for expression of CMV gene *UL55* (glycoprotein B) as a marker of CMV presence in the tumor samples. The results of a sensitive nested reverse transcriptase polymerase chain reaction (RT-PCR) reaction for expression of CMV gene *UL55* are depicted. The distribution of molecular subtypes of GBM histotypes among the 40 tumor samples is denoted above each bar graph.

Chapter 3:

gB-specific CAR T cells exhibit anti-tumor activity against HCMV-positive glioblastoma multiforme tumors

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Abstract

Glioblastoma multiforme (GBM) is the most common and deadliest adult primary brain tumor. Immunotherapeutic approaches using chimeric antigen receptor (CAR) T cells have shown limited efficacy against GBM due in part to heterogeneous target antigen expression. Human cytomegalovirus (HCMV) can be detected in up to 90% of GBM samples but not in the surrounding normal brain tissue. We hypothesized that the presence of HCMV within GBM presents a novel approach by which T cells can be redirected to GBM. We generated a CAR optimized to recognize the HCMV surface antigen glycoprotein B (gB). *In vitro* testing of the anti-gB CAR T cells revealed specific activity against glioma cells expressing gB and HCMV-infected cells. Importantly, gB CAR T cells were able to recognize *ex vivo* cultured primary GBM tumors. Finally, gB CAR T cells exhibited anti-tumor activity *in vivo* against GBM tumors in a xenograft mouse model. These results suggest that CAR T cells may be effective in recognizing HCMV antigens found within GBM. Taken together, the results of this study show the feasibility of using gB CAR T cells as a platform to target HCMV in GBM.

Introduction

Chimeric antigen receptors (CAR) are synthetic gene constructs that most commonly combine the specificity of monoclonal antibodies with the signaling domains of co-stimulatory molecules and TCR zeta chains. CARs can be used to redirect T cells to surface antigens by changing the specificity of the recognition domain. This approach has been used successfully in the use of CAR T cells to treat hematological malignancies leading to the first clinical approval of a CAR T cell therapy for acute lymphoblastic leukemia (Maude et al., 2014). However, in the realm of solid tumors CAR T cells have shown limited efficacy, in part due to the heterogeneous expression of target antigen and the associated safety profile of targeting tumor-associated antigens. Target antigens on solid tumors offer a special problem in that often tumor-associated antigens are found on normal tissue of vital organs or have heterogeneous expression on the tumor cells. This problem is particularly challenging in the development of CAR T cell therapies directed against glioblastoma (GBM).

GBM is an intractable tumor with a grim prognosis. Median life expectancy with current standard of care is approximately 15 months. Current standard of care consists of surgical resection and concomitant radiation and chemotherapy (Stupp et al., 2005; Wen & Kesari, 2008). The current modalities used to treat GBM are imprecise and often do not prevent tumor recurrence. Adoptive transfer of gene-engineered T cells offers greater specificity for tumors than conventional therapies but has had limited success in clinical trials against GBM. A clinical trial using CAR T cells re-directed to EGFRvIII led to a reduction of EGFRvIII-expressing tumor cells but did not cause significant tumor reduction, potentially due to antigenic heterogeneity (O'Rourke et al., 2017). CAR T cells targeting IL13Rα2 in a phase I clinical trial showed tumor regression, a 9-month remission, and relapse with antigen-negative GBM in one patient (Brown et al., 2016). These studies highlight the challenges of targeting GBM, which displays significant heterogeneity in

antigen expression and the need to identify additional target antigens present within GBM (deCarvalho et al., 2018; Francis et al., 2014; Little et al., 2012).

Human cytomegalovirus (HCMV) is a tumor-associated antigen that has been detected in up to 90% of GBM cases (Mitchell et al., 2008). The mechanism of HCMV expression in GBM remains unknown but HCMV proteins have been reported to enhance tumorigenicity (Dziurzynski et al., 2012). HCMV antigen glycoprotein B (gB) has been shown to be expressed in primary GBM and contribute to GBM invasiveness in mouse models (C. Cobbs et al., 2014). gB is a highly conserved fusogenic protein found on the HCMV envelope known to assist HCMV in viral entry into host cells (Gardner & Tortorella, 2016). During HCMV lytic infection, surface expressed gB aids HCMV in cell-to-cell spread (Isaacson & Compton, 2009). Moreover, gB is one of the most abundant extracellular HCMV antigens and thus potentially available to CAR T cell recognition (Varnum et al., 2004). Evidence for targeting HCMV proteins as a tumor-associated antigen in GBM comes from clinical trials using dendritic cell vaccines. In one clinical trial, autologous dendritic cells pulsed with GBM tumor lysate led to the expansion of HCMV-specific T cells in one patient (Prins et al., 2008). Another study used HCMV antigen pp65 mRNA pulsed into autologous dendritic cells with tetanus toxoid adjuvant, leading to prolonged survival in patients with GBM (Mitchell et al., 2015). Lastly, a study conducted by Nair *et al*. demonstrated that HCMV-specific T cells expanded from patients with GBM were able to recognize the patient's autologous GBM, adding to the rationale to target HCMV in GBM (Nair et al., 2014).

The high prevalence of HCMV in GBM tumors suggests that T cells could be re-directed to recognize and kill HCMV-infected glioma cells. HCMV is a foreign virus with high immunogenicity allowing for generation of strong immunological responses to its antigens and potentially to GBM. We set out to determine the feasibility of generating an

anti-HCMV CAR that would recognize and kill GBM tumors expressing HCMV surface antigen gB.

Results

Epitopes of HCMV gB

The human donor derived mAb ITC52 was isolated from immortalized B cells and was determined to be specific for gB antigen domain 1 (AD-1). Precise epitope mapping of ITC52 revealed recognition of a discontinuous epitope in AD-1 corresponding to two residues 570-579 and 606-619 of HCMV gB (Ohlin, Sundqvist, Mach, Wahren, & Borrebaeck, 1993). Moreover, alignment of the amino acid sequences of 60 different HCMV strains shows a high degree of amino acid conversation among strains in AD-1 (Figure 3.1). A common substitution mutation found in gB AD-1 corresponding to residue F611L does not prevent recognition of both variants of gB (Speckner et al., 1999). These data suggest that a gB-specific CAR bearing the ITC52 scFv would have near universal recognition of all HCMV strain specific gB variants.

Design and optimization of anti-HCMV gB CAR T cells

Based on the observed expression of CMV antigen IE1-72 within primary GBM FFPE samples (Fig.2.1) we next set out to design a chimeric antigen receptor (CAR) that can detect membrane-bound HCMV glycoprotein B (gB) antigen reported to be expressed in GBM (C. Cobbs et al., 2014). We generated a second-generation CAR that incorporated the CD137 (4-1BB) co-stimulatory domain with TCR zeta signaling (Milone et al., 2009) (Fig. 3.2 A). The scFv used was derived from the variable domains of the human monoclonal antibody ITC52, which recognizes the membrane distal epitope AD-1 found

on gB (Burke & Heldwein, 2015; Heldwein et al., 2006; Schoppel et al., 1996). The scFv was generated in two orientations with the variable heavy and light chains connected via a 3x(GGGGS) spacer domain onto a CD8α hinge domain (Fig. 3.3 A). To determine the optimal scFv orientation, the CAR constructs were evaluated utilizing multiple immunoassays against the human glioma tumor cell line, U87, which was lentivirally transduced to stably express gB (Fig. 3.2 A). HCMV gB is not naturally expressed in U87 and ectopic expression of the target antigen allows specificity determination of gB CAR constructs. Cytokine production, as determined by intracellular cytokine staining (ICCS) and flow cytometry, was higher in the T cells bearing the CAR with scFv orientation of light to heavy (L2H) as compared to T cells bearing the CAR with H2L scFv orientation (Fig. 3.2 B). The L2H orientation also showed significantly greater cytolytic function by ^{51}Cr-release assay (Fig. 3.2 C).

We further optimized the design of the anti-gB CAR by testing various hinge domains (Fig. 3.3 A). We hypothesized that there may be an optimal hinge length between the apical AD-1 epitope found on gB and our CAR expressing T cells as demonstrated previously in other CAR models (Hudecek et al., 2013; S. E. James et al., 2008). We generated three additional gB CAR constructs using the long IgG4 hinge, IgG4/2NQ mutant hinge, and a short 12 amino acid hinge derived from the dimerization core of the IgG4 Fc domain (Hudecek et al., 2015) (Fig.3.3 A). The four gB L2H CAR constructs were tested via ICCS for production of IFN-γ, TNF-α, IL-2, and CD107a. CAR T cells expressing the short 12aa hinge domain exhibited the highest levels of cytokine production and CD107a marker of degranulation, consistent with a report of CAR T cells targeting the apical epitopes of ROR1 using a short spacer domain (Fig. 3.3 B) (Hudecek et al., 2013). Interestingly, the cytolytic activity of the three CAR constructs differed from their cytokine production activity. CAR T cells bearing the mutated IgG4/2NQ domain displayed higher cytolytic activity in a 6-hour ^{51}Cr release assay (Fig. 3.3 C). Based on the divergent *in vitro*

effector activities of the short (12aa) and long hinge (IgG4/2NQ) gB CARs we selected these two CAR constructs for further evaluation.

The initial *in vitro* testing of our CAR constructs relied upon the transduced U87 glioma cell line expressing artificially high levels of gB antigen (Fig.3.2 A). We set out to evaluate how gB CAR T cells respond to cells naturally infected with HCMV. gB expression during the course of infection undergoes a dynamic process, where antigen is initially trafficked to the surface of infected cells and then endocytosed to an intracellular viral envelopment compartment (Radsak et al., 1996). We tested the ability of anti-gB CAR T cells to respond to human foreskin fibroblasts (HFF) infected with HCMV. Expression of gB was monitored in HCMV-infected HFFs over the course of five days (Fig.3.4A). On day four post infection, gB CAR T cells were co-cultured with mock infected or infected HFF cells. gB CAR T cells were able to detect gB expressed on HCMV-infected HFF cells at levels comparable to positive control targets (Fig.3.4 B). Moreover, the pattern of recognition was similar to that previously observed in U87gB, with the short hinge gB 12aa CAR exhibiting the highest cytokine production. The ability of anti-HCMV CAR T cells to produce high levels of cytokines is a desirable feature for antiviral efficacy as another group has shown that TNF-α and INF-γ can inhibit HCMV replication in infected HFF cells (Proff, Brey, Ensser, Holter, & Lehner, 2018).

gB CAR T cells recognize cognate antigen on some primary GBM tumors

It is important to demonstrate that the gB CAR T cells can recognize the natural level of gB antigen expression found in primary GBM tumors. Low levels of target antigen expression on tumor cells could hinder the ability of CAR T cells to recognize and kill antigen-positive tumor cells (Walker et al., 2017). Previous studies using autologous HCMV-specific T cells co-cultured with the patient's own tumor revealed the ability of T cells to sense endogenous levels of HCMV antigen within GBM tumors, presumably by

the endogenous CMV-specific TCR (Nair et al., 2014). However, despite demonstrating gB expression within GBM, levels of target antigen may be below the level of detection for CAR T cells (C. Cobbs et al., 2014; Dziurzynski et al., 2011; Mitchell et al., 2008). To test the ability of gB CAR T cells to recognize their cognate antigen on primary GBM tumors, we cultured short-term newly resected GBM tumors as targets in a ^{51}Cr release assay. To promote retention of HCMV within primary GBM tumors, the cells were cultured in glioma-stem cell promoting conditions (Fiallos et al., 2014). Glioma cell line U87 and U87gB were used as negative and positive control targets, respectively, to monitor CAR T cell activity (Fig.3.5A). One of the three primary tumors tested, tumor sample GBM-8017, was killed at low effector-to-target ratios when co-cultured with gB CAR T cells and not with negative control CD19 CAR T cells or untransduced T cells, excluding an allogeneic effect (Fig. 3.5B). The other two primary GBM cells were not specifically lysed by gB CAR T cells. In contrast, the EGFR CAR T cells specifically killed the two GBM explants that were not lysed by the gB CAR T cells. We later confirmed expression of UL55 (gB) in GBM8017 via RT-PCR. These data support the hypothesis that gB CAR T cells are sensitive enough to detect extracellular expression of gB in some tumors at levels of natural HCMV infection within primary GBM tumor cells.

gB CAR T cells delay GBM tumor growth in vivo

Based on the observed anti-tumor activity of the gB CAR T cells in vitro we next sought to evaluate the impact of gB CAR T cell on tumor growth *in vivo*. High levels of *in vitro* activity could compromise the *in vivo* function of CAR T cells via induction of activation induced cell death (Kunkele et al., 2015). We tested the short (12aa) and long hinge (IgG4/2NQ) gB CAR constructs in an established human tumor xenograft model using NSG mice bearing subcutaneous tumors expressing gB (U87gB). Five days after tumor injection, a single injection of $5x10^6$ CAR+ T cells was administered via tail vein (Fig.3.6A). All mice

treated with CAR T cells demonstrated tumor engraftment and progression. However, a significant reduction in tumor growth was observed in mice treated with either anti-gB CAR T cell as compared to negative control CD19 CAR T cell treated mice (Fig.3.6B,D). All mice treated with control CD19 CAR T cells succumbed to their tumor by day 30, while the gB CAR treated mice had a significant survival advantage (Fig. 3.6C). Surprisingly, despite both short and long versions of the gB CAR T cells displaying different *in vitro* activity, mice in both gB CAR T cell treated groups had a similar delay in tumor growth and a gain in survival *in vivo* (Fig.3.5C).

CAR T cells recognize endogenous levels of HCMV gB expression in primary GBM tumor explants

Having tested our anti-gB CAR T cells *in vivo* in a high antigen expressing pre-clinical model, we next evaluated the anti-tumor effect of gB CAR T cells in a low antigen expressing model. We screened human GBM explants for HCMV expression and found expression of target antigen gB in the human glioma explant D270. Despite being able to detect expression of gB in this cell line at the mRNA level, gB levels were below the level of antibody detection by flow cytometry (Fig 3.7A). Using a secondary marker for HCMV, we detected the expression of HCMV IE1-72 using a sensitive IHC protocol (Fig.3.7B). Surprisingly, low level gB expression was sufficient to induce potent and specific killing of D270 tumor cells when co-cultured with gB CAR T cells in ^{51}Cr-release assays (Fig.3.7C). D270 cells have been shown previously to express EGFRvIII, and EGFRvIII CAR T cells were also effective. We next sought to evaluate the antitumor function of gB CAR T cells against D270 in a subcutaneous murine tumor model. We reasoned that D270 would be a realistic surrogate to evaluate the antitumor function of gB CAR T cells expressing natural levels of HCMV gB expressed in primary GBM tumors. Tumor bearing mice were treated with two doses of $5x10^6$ CAR+ T cells, at day 3 and day 10 (Fig.3.8A). Mice treated with negative control CD19-specific CAR T cells demonstrated tumor growth comparable

to PBS treated mice (Fig.3.8B). In contrast, gB CAR T cell treated mice had significantly reduced tumor growth (Fig.3.8B). Interestingly, a small subset of gB CAR T cell treated mice controlled their tumors and displayed stable disease (Fig.3.8C). Taken together, anti-gB CAR T cells lyse tumor cells expressing low levels of gB on the cell surface and have significant antitumor efficacy *in vivo*.

Materials and Methods

Cell lines and cell culture

The human glioma cell lines U87 and U87-EGFRvIII were grown under conditions reported in Johnson et al. (2015). The U87-EGFRvIII parental cell line was lentivirally transduced to express HCMV gene *UL55* (gB) and single cell cloned in flat-bottom 96-well plates. Human glioma xenograft explant D270 was provided by Darell Bigner (Duke University, Durham, NC). All glioma cell lines were grown in Improved MEM Zinc Option (Gibco) supplemented with 10% fetal calf serum (Seradigm), 1% penicillin and streptomycin (Gibco), 1% L-Glutamax (Gibco), 1% Sodium Pyruvate (Gibco), and HEPES buffer (20mM) (Gibco). Primary GBM tumors and cell lines were grown as described in (Roccograndi et al., 2017). GBM tissue was collected with informed patient consent, under a protocol approved by the University of Pennsylvania's IRB. All samples were de-identified before processing. Human foreskin fibroblasts (HFF) were mock infected or infected with TOWNE strain HCMV virus at a MOI of 5 to ensure close to 100% infection of target cells. Human glioma cell line U87 and U87gB was authenticated by the University of Arizona to confirm identify. D270 and primary HFF cells were screened to rule out cross contamination with other tumor cell lines. All cell lines used were tested and confirmed to be mycoplasma negative using MycoAlert Mycoplasma Detection Kit (Lonza).

Generation of CAR constructs

Anti-gB CARs were generated from a pTRPE 5E5-CD8a-BBz and pTRPE 5E5-IgG4-BBz construct (Posey et al., 2016). These constructs were digested overnight using BspEI and BamHI restriction enzymes and gel extracted. Amino acid sequences of ITC52 scFv were found on GenBank accession L26537 and L26538. Anti-gB single chain variable fragment (scFvs) were codon optimized for human expression and synthesized (Geneart, gBlock IDT) to contain the BspEI and BamHI restriction sites. Digested product was then ligated into pTRPE 5E5-CD8a-BBz and pTRPE 5E5-IgG4-BBz. Ligation products were transformed using Stbl3 chemically competent cells to prevent recombination of lentiviral vector. Hinge domains for IgG4/2NQ hinge were generated using site-directed mutagenesis using primers listed in Hudecek et al. (2015). The mutated hinge domain was then swapped into parental pTRPE ITC52 L2H IgG4 BBz digested with EcoRV and SalI restriction enzymes. 12aa hinge domain was made using oligonucleotides containing BamHI and EcoRV for amplification from pTRPE ITC52 L2H IgG4/2NQ BBz using the following primers: 1) 5'-GGATCCGAAATCGTTCTGACCCAG-3', 2).5'-GATATCAGGGCAAGGG-3'. The truncated hinge was then ligated into digested pTRPE backbone containing pTRPE ITC52 L2H. Generation of CD19 CAR was previously described in (Milone et al., 2009), EGFR CAR in (Wing et al., 2018), and EGFRvIII CAR in (Johnson et al., 2015), respectively.

Transduction of T cells and expansion

Normal donor leukocytes were isolated from leukapheresis product from de-identified normal donors by the Human Immunology Core of the University of Pennsylvania. T cells were stimulated with CD3/CD28 Dynabeads Human T-Activator (Life technologies) at a bead-to-cell ratio of 3:1. T cells were cultured in R10 media (RPMI 1640 medium supplemented with 10% fetal calf serum (Seradigm), 1% penicillin and streptomycin, 1%

L-Glutamax and HEPES buffer (20mM) (Gibco). IL-2 was added to the culture media at a final concentration of 30U/ml. Growth and expansion of the stimulated T cells was monitored using a Coulter Multisizer measuring cell density and mean lymphocytic volume and cells were frozen when T cells were rested (mean volumes of 300-330 fL).

Intracellular cytokine analysis

CAR T cells and untransduced T cells were cultured with target cells at an effector-to-target ratio of 1:1. % CAR T cells were normalized to lowest percent transduction using untransduced cells. Effector T cells and tumor target cells were counted and resuspended at a density of $2x10^6$/mL and seeded into a round bottom 96-well plate in 100μL. Co-culture R10 media contained Golgi-stop and Golgi-plug (both BD Bioscience). Staining and processing of co-culture T cells was done as described in Johnson et al. (2015). Cells were analyzed on LSRII with gating on live, singlet CD3+ lymphocytes.

Cytolytic assay

T cell killing was determined using standard ^{51}Cr release assay. ^{51}Cr release assay used targets that were labeled for up to two hours at 37°C with 50 μ CI of radioactive ^{51}Cr. Labeled cells were washed twice in 10mL of non-phenol red culture media supplemented with 5% FBS and resuspended at a $1x10^5$ cells/mL. One hundred microliters of labeled target cells were added to round bottom 96-well plate. Effector T cells were added to target cells in 100μL at different effector-to-target ratios. Absolute T cell count was normalized across all effector groups with the addition of untransduced T cells. The co-culture was run for up to 6-hours at 37°C. Thirty-five microliters of the supernatant was collected and transferred onto the filter of LumaPlate. The transferred supernatant was dried overnight on filter paper. Cytolytic activity of effector T cells was measured using a beta-emissions liquid scintillation counter (Perkin Elmer). Percent specific lysis was determined using the

following calculation: (sample counts-spontaneous counts)/(maximum counts-spontaneous counts) x100.

Murine Models

NOD SCID gamma delta (NSG) mice were purchased from approved vendors (Jackson labs) and housed in a contained area. All mouse experiments adhered to Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. In the U87gB-EGFRvIII model, we followed the protocol established in Johnson et al. (2015). Tumor growth was evaluated via caliper measurement at regular intervals. Endpoint was determined by a tumor measuring 2cm in any dimension (length, width, height), loss of 15% body weight, or inability to ambulate. In the D270 subcutaneous model, NSG mice were inoculated with $4x10^5$ D270 cells in the mouse flank. Three days after tumor engraftment, mice were treated with CAR T cells at a dose of $5x10^6$ CAR+ T cells per dose via tail-vein injection and again at day 10 after tumor injection. Absolute T cell count was matched for all groups with untransduced T cells.

Statistical Methods

Data is reported as means ± SD or SEM or as stated in figure legends. Statistical significance was determined using a standard two-way ANOVA for *in vitro* assays. Survival advantage for *in vivo* tumor challenges was determined using log-rank Mantel-Cox. P values of less than 0.05 were considered statistically significant. All statistical analyses were performed with Prism software version 8.0 (GraphPad).

Discussion

The primary objective of this study was to determine if HCMV antigen gB could serve as a viable tumor-associated antigen for CAR T cells. The high prevalence of the virus in GBM samples make HCMV an attractive TAA (C. S. Cobbs et al., 2002; Mitchell et al., 2008). The ability of CAR T cells to recognize their cognate antigen in an HLA-independent manner allows for a potentially broad clinical application of anti-HCMV CAR T cell therapy for most cases of GBM. gB is one of many potential HCMV-associated targets that been reported to be expressed within GBM that can be used to redirect T cells via CAR or TCR gene transfer (reviewed in (Rahman et al., 2019)). Moreover, HCMV antigens are readily recognized by the immune system due to their foreign nature and could increase the likelihood of inducing epitope spreading, which could benefit patients with GBM (Terrazzini & Kern, 2014).

CAR T cells re-directed to gB have been previously reported (Full et al., 2010; Proff et al., 2018); the gB CARs in those reports were used to study the effects of retargeted T cells on acute infection with HCMV. However, to our knowledge the present work is the first report demonstrating that gB CAR T cells have promise as a therapeutic for GBM. Optimization of our anti-gB CAR underwent two phases, determination of the most sensitive orientation of the variable heavy and light fragments and selection of a hinge domain that allows more effective CAR T cell interaction with its cognate epitope. The epitope of ITC52 has been mapped to a membrane distal location atop of the gB trimer (Burke & Heldwein, 2015; Heldwein et al., 2006). Other groups have found that the avidity of the scFv and the location of the epitope on the cognate antigen has an effect on the efficacy of the CAR (Hudecek et al., 2013; S. E. James et al., 2008). These findings were consistent with our results in that the short, truncated hinge (gB 12aa) had the highest cytokine production.

Our results demonstrated *in vivo* antitumor efficacy of gB CAR T cells in two aggressive tumor models. Most strikingly, we observed antitumor efficacy in the D270 tumor model. This was notable because of the low level of surface expression of gB, which was below the limit of detection by flow cytometry. CAR T cells, depending on the CAR construct and epitope that is targeted, have varying levels of sensitivity. For CD19, CAR T cells have been reported to require several hundred targets per tumor cell (Stone, Aggen, Schietinger, Schreiber, & Kranz, 2012), and more recently, a threshold for CAR recognition below 100 CD19 molecules per cell was reported (Nerreter et al., 2019). Thus, CAR T cells in optimal conditions can recognize targets at levels that are well below detection by flow cytometry. For potential application of CAR T for therapy of GBM, sensitive recognition of target will be important given the consensus that HCMV is expressed at low levels in GBM.

Targeting HCMV within GBM has shown some signs of promise in the clinic using TCR-based immunotherapies that target HCMV (Ahmed et al., 2017; Batich et al., 2017; Ghazi et al., 2012; Mitchell et al., 2015; Prins et al., 2008; Schuessler, Walker, & Khanna, 2014). In a series of clinical studies using dendritic cell vaccines pulsed with pp65 in conjunction with adoptive transfer of HCMV-specific T cells, patients saw a restoration of poly-functionality in the adoptively transferred HCMV-specific T cells (Reap et al., 2018). An alternative approach to overcome the inherent barriers of low frequency or exhaustion of endogenous HCMV-reactive T cells is to use a gene-engineered approach to redirect polyclonal T cells to HCMV antigens. On this note, a report investigating tumor infiltrating T cells found HCMV-specific T cells to be present within GBM tumors; however, these cells displayed a tolerized and exhausted phenotype (Bahador et al., 2017). This study suggests that endogenous T cells primed via HCMV infection recognize HCMV antigen in the tumor but have a limited impact on tumor growth potentially due to the development of an exhausted phenotype. CAR T cells may fair better in the tumor microenvironments

but may be limited in their ability to clear all tumor due to heterogeneous expression of target antigens within GBM tumors (C. S. Cobbs et al., 2002; O'Rourke et al., 2017).

One limitation of our study is the inability to address the impact of inducing a recall response to HCMV in gB CAR T cell treated mice. Human cytomegalovirus is species-specific and cannot infect other animal hosts; therefore, we were unable to interrogate the ability of endogenous polyclonal HCMV-specific T cells to be recalled and aid in the destruction of glioma cells infected with HCMV. A more pressing issue that gB CARs may face is the inability of a single scFv to recognize gB variants from clinical strains of HCMV due to mutations in the target antigen, potentially resulting in escape from detection by CAR T cells. Loss of recognition of the AD-1 epitope of the ITC52 monoclonal antibody has been reported. Small amino acid substitutions in the AD-1 epitope of gB lead to loss of neutralizing activity of AD-1 binding antibodies (Schoppel et al., 1996; Speckner et al., 1999). How comparable mutations in clinical strains would compromise the ability of CAR T cells to recognize their target remains to be evaluated. Two modes of administering CAR T cell products to patients have been evaluated clinically. In a clinical trial using CAR T cells directed to EGFRvIII, patients were given CAR T cells in a single IV infusion. CAR T cells were able to traffic to the brain and kill EGRFvIII positive cells (O'Rourke et al., 2017). CAR T cells re-directed to IL13RA2 were given multiple doses intracranially over the course of week and demonstrated tumor regression in one case (Brown et al., 2016). Both modes of T cell administration allow T cells to perform anti-tumor function; however, it remains to be determined if one mode of delivery would have a greater clinical impact over another using the same CAR T cells administered through both routes.

An alternative use of gB CAR T cells that we have developed is in the context of HCMV reactivation (Full et al., 2010; Proff et al., 2018). Adoptive cell transfer of viral-specific T cells to control viral reactivation has been tested clinically with great success (Harris, Davila, Bollard, & Keller, 2019; Papadopoulou et al., 2014; Riddell et al., 1992).

These clinical trials demonstrate the feasibility of using cell transfer in controlling viral reactivation; however, to our knowledge and with the exception of HIV, no clinical trials have reported the use of CAR T cells as the modality of treatment for viral infection (Scholler et al., 2012). CAR T cells overcome a major limitation of ex-vivo expanded viral-specific T cells by recognizing their cognate antigen in an MHC-independent fashion. Circumnavigating HLA-restriction allows for broad clinical application of virus-specific CAR T cells for patients with compromised or suppressed immune systems experiencing viral reactivation (Gandhi & Khanna, 2004; Jain et al., 2014).

The use of CAR T cells simultaneously targeting multiple tumor antigens in GBM is a logical next step to overcome tumor heterogeneity. This approach is already being explored via the development of a trivalent CAR construct that simultaneously targets three tumor-associated antigens found in GBM (Bielamowicz et al., 2018). Additionally, CAR T cells redirected to IL13RA2 (Brown et al., 2016) and EGFRvIII (O'Rourke et al., 2017) have proven to be safe and tolerated well by patients in clinical trials. Given the high prevalence of HCMV in GBM (C. S. Cobbs et al., 2002; Mitchell et al., 2008), the use of a gB CAR T cell in conjunction with other GBM CAR T cell targets could decrease tumor escape by limiting the emergence of antigen loss variants.

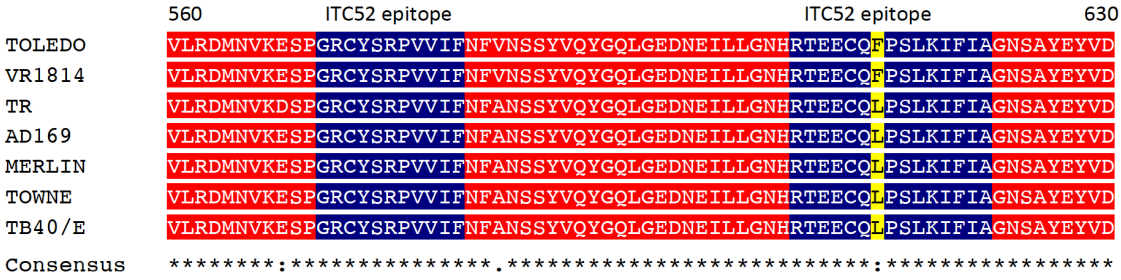


Figure 3.1. Sequence alignment of gB from clinical and lab-adapted strains of HCMV. Conserved residues among strains are highlighted in red; the dissentious epitope of mAb ITC52 is highlighted in blue; highlighted in yellow is L611F, the only residue substitution found in the epitope of ITC52. AD169 and Towne represent lab-adapted strains. Toledo, VR1814, TR, Merlin, and TB40/E are representative clinical strains. HCMV gB sequences were exported from NCBI's RefSeq or Uniport database. Sequence alignment was done using Clustal Omega.

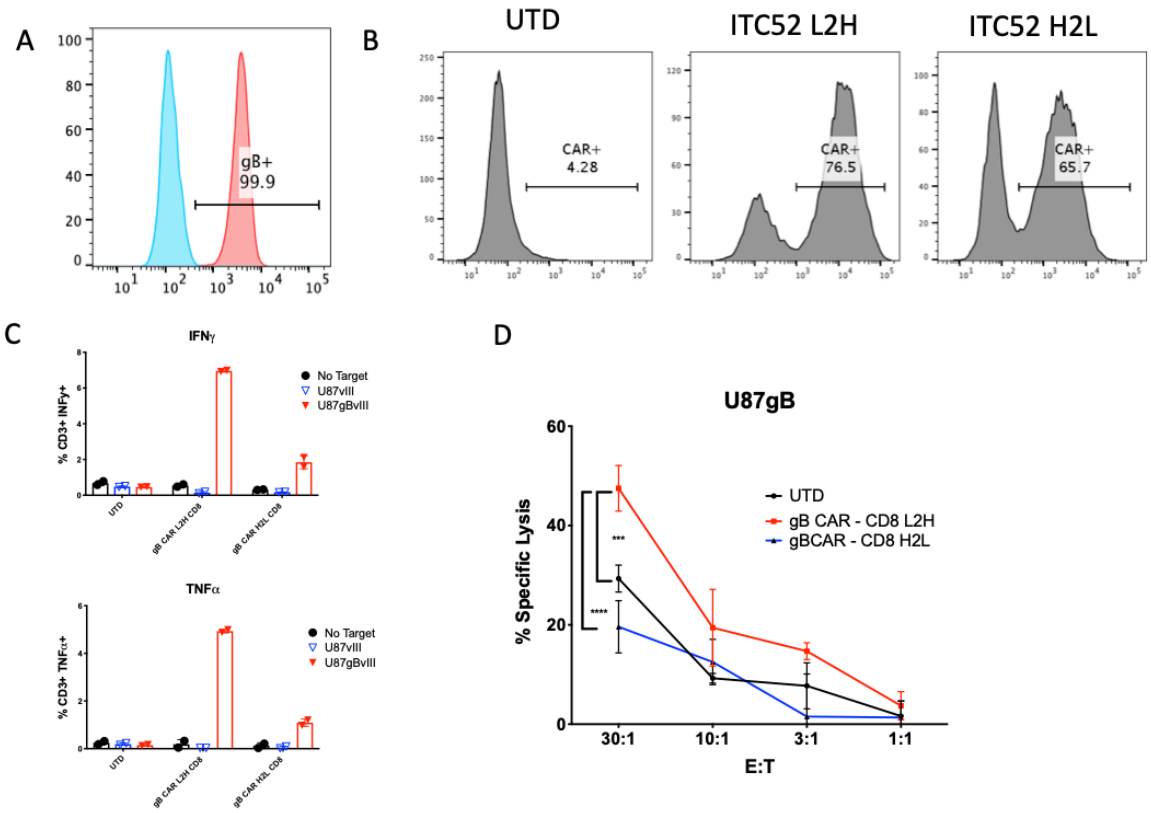


Figure 3.2. A. Expression of glycoprotein B (gB) on U87 human glioma cell transduced with gB antigen. The cell lines were immunostained with anti-gB monoclonal and analyzed via flowcytometry. Parental cell line U87 is shown in blue and U87gB shown in red. **B.** Expression of anti-gB CAR on transduced T cells. CAR T cells were immunostained with anti-human fab-biotin and PE-streptavidin secondary and analyzed via flow cytometry. CARs with different heavy and light chain orientation in the scFv were tested. **C.** The function of gB CARs with L2H or H2L orientation was assessed by production of tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ) via an intracellular cytokine-staining assay. Untransduced (UTD) control T cells and gB CAR expressing T cells from the same donor were co-cultured with human glioma cell line U87 or with U87 cells transduced with gB at an effector to target ratio of 1:1 overnight. The complete panel of gB CARs was tested in two independent donors and representative results shown. **D**. Cytolytic activity of gB CAR T cells was monitored using a 6hr chromium release assay. Shown are representative results for one donor. This assay was performed at least twice with 2 independent donors. Plots show means +/- SEM of triplicate wells. Statistical analysis was done using two-way ANOVA for multiple comparisons with Tukey's correction (* = $p<0.05$, *** = $p<0.0005$).

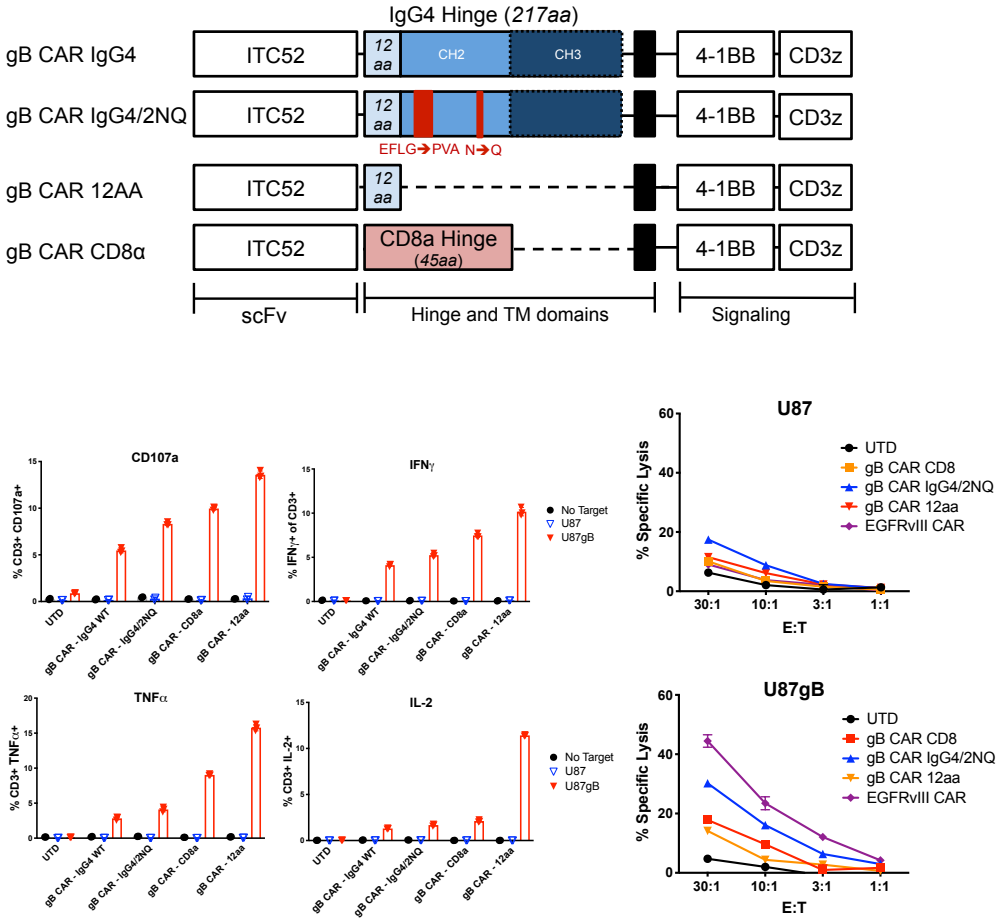
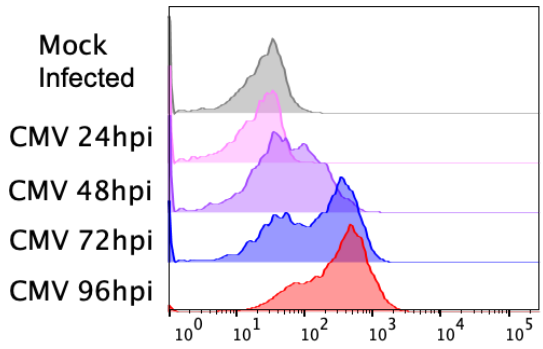


Figure 3.3. CAR T cells redirected to CMV gB exhibit antigen-specific activity. **A**. Vector map of anti-gB CAR constructs designed with different spacer domains of various lengths. Highlighted in red are mutations introduced into the gB IgG4 CAR variant to reduce Fc receptor binding in vivo. **B.** Production of tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ), interleukin-2 (IL-2) and a marker of degranulation (CD107a) was assessed via an intracellular cytokine-staining assay. Untransduced (UTD) T cells and gB CAR expressing T cells from the same donor were co-cultured with human glioma cell line U87 or with U87 cells transduced with gB at an effector to target ratio of 1:1 overnight. The complete panel of gB CARs was tested in two independent donors and representative results are shown. **C.** Cytolytic activity of gB CAR T cells was monitored using a 6hr chromium release assay. Shown are representative results. This assay was performed at least twice with 2 independent donors. Plots show means +/- SEM of triplicate wells.

A.



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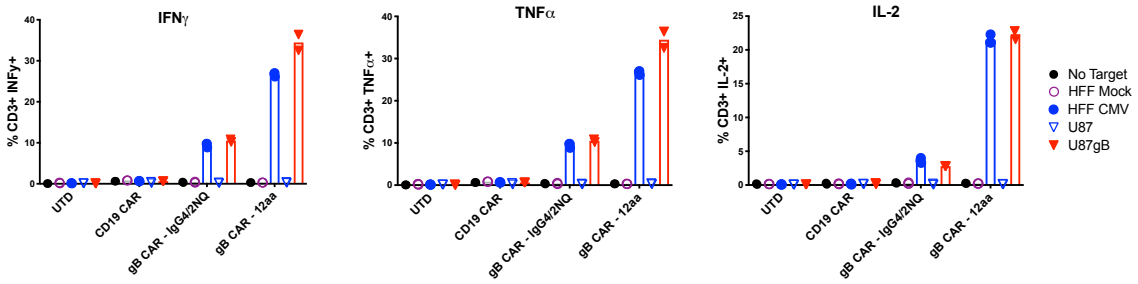

Figure 3.4. A. CMV-infected human foreskin fibroblasts (HFFs) were co-cultured with gB CAR T cells. Production of TNFα, IFNγ, and IL-2 monitored via an intracellular cytokine-staining assay. Untransduced (UTD) T cells and gB CAR expressing T cells from the same donor were co-cultured with HFFs mock-infected or infected with HCMV 96 hours post infection at an effector to target ratio of 1:1 during overnight co-culture. This experiment was performed twice using a total of three independent donors. Statistical analysis was done using a two-way ANOVA with multiple comparisons using Tukey's correction (* = $p<0.05$, ***= $p<0.0005$). **B.** Human foreskin fibroblasts were infected with HCMV at an MOI of 1:5 or mock infected. Infected cells were intracellularly stained with anti-gB monoclonal and analyzed by flow cytometry for gB expression over a five day time course post infection. Statistical analysis was done using two-way ANOVA for multiple comparisons with Tukey's correction (* = $p<0.05$, *** = $p<0.0005$).

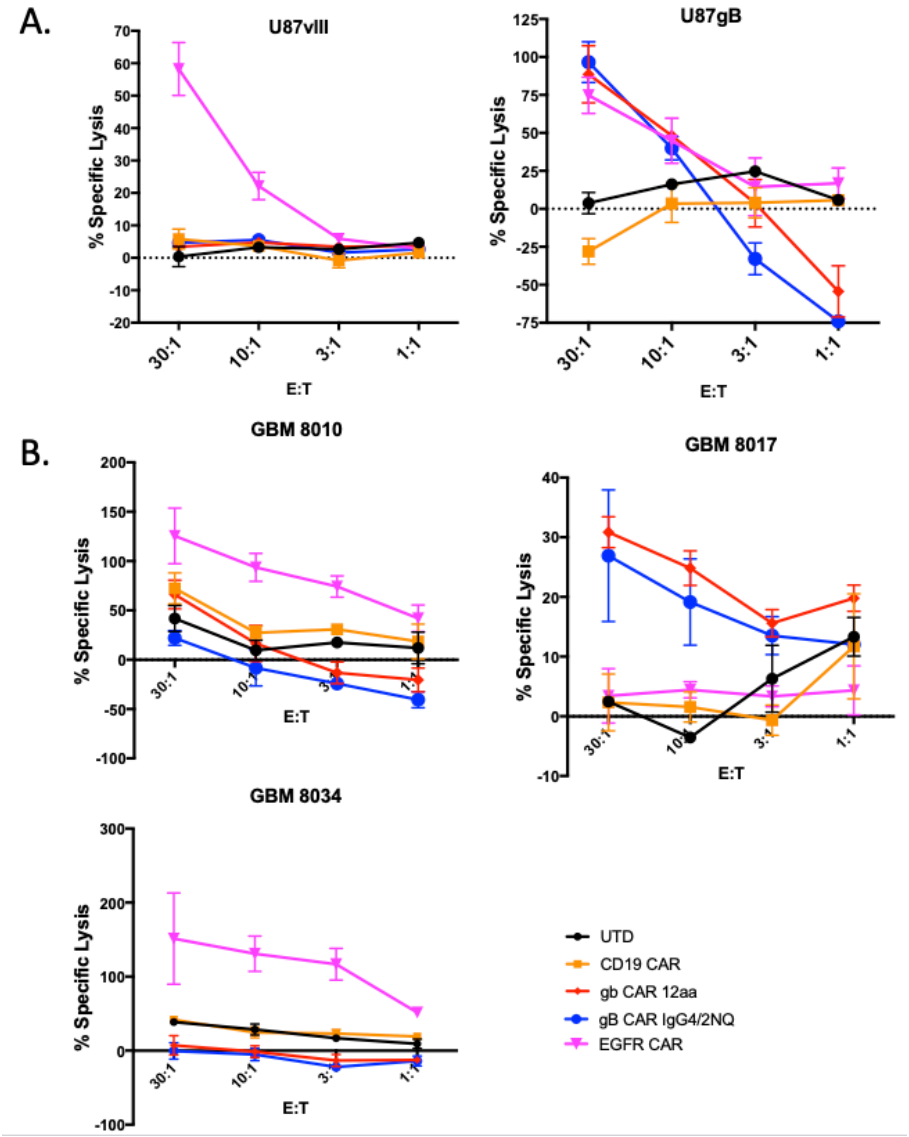

Figure 3.5. gB CAR T cells kill GBM cell lines (**A**) and tumor explants (**B**) cultured ex-vivo. **A.** Glioma cell lines U87 and U87gB (4hr time point) and **B.** ex-vivo cultured GBM tumors (6hr time point) were co-cultured with effector T cells at effector:target ratios ranging from 30:1 to 1:1. Newly resected GBM tumors were cultured for up to 20 days post resection for these experiments. A chromium release assay was used to measure specific lysis of ex-vivo cultured tumors. Effector to target ratio is based on CAR + T cells and total T cell count was normalized among control groups. Negative controls were untransduced T cells (UTD) and CD19 CAR T cells. EGFRvIII CAR T cells were used as a general positive control for most experiments. Plots show means +/- SEM of triplicate wells. Shown are the results of one assay using tumor cells from three different patients co-cultured with one allogeneic T cell donor.

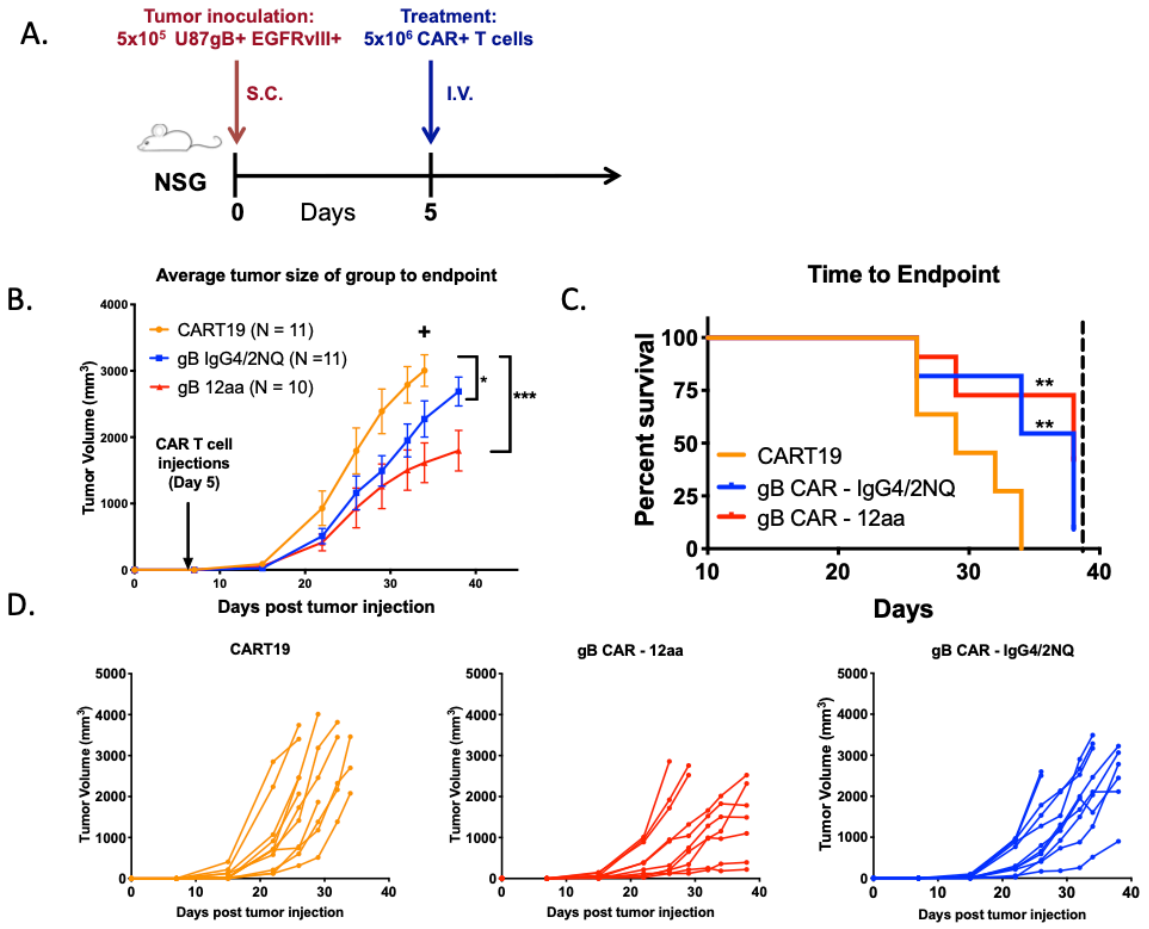

Figure 3.6. Antitumor effects of gB CAR T cells in vivo. **A.** Schematic representation of a murine subcutaneous tumor model using NOD scid gamma (NSG) mice. NSG were inoculated subcutaneously with $5x10^5$ U87gB+ tumor cells (n=10 or more animals per group). Five days after tumor engraftment, mice were randomized based on tumor bioluminescence and treated with $5x10^6$ CAR+ T cells per mouse via tail vein injection. The addition of untransduced T cells to the total T cell dose was used to normalize the total amount of T cells given per mouse. **B.** The predetermined endpoints were determined by serial caliper measurements taken at regular intervals until the tumor reached the endpoint of 2cm in any dimension (LxWxH) or the mouse showed signs of graft versus host disease (GHVD). + Indicates all mice have reached the predetermined humane endpoint. Each line represents the mean average of the group and standard error of the mean represented by the error bars. A two-way ANOVA was performed comparing experimental gB CAR T cells to the CD19 CAR T cell treated mice (* = $p<0.05$, *** = $p<0.0005$)). **C.** Survival based on time to endpoint of tumor-bearing mice treated with gB CAR T cells or CD19 CAR T cells. Kaplan-Meier curve and statistical significance between experimental and control groups determined using log-rank Mantel-Cox test. **D.** Individual growth curves for each mouse per group until reaching endpoint. The experiment was repeated twice with two independent T cell donors.

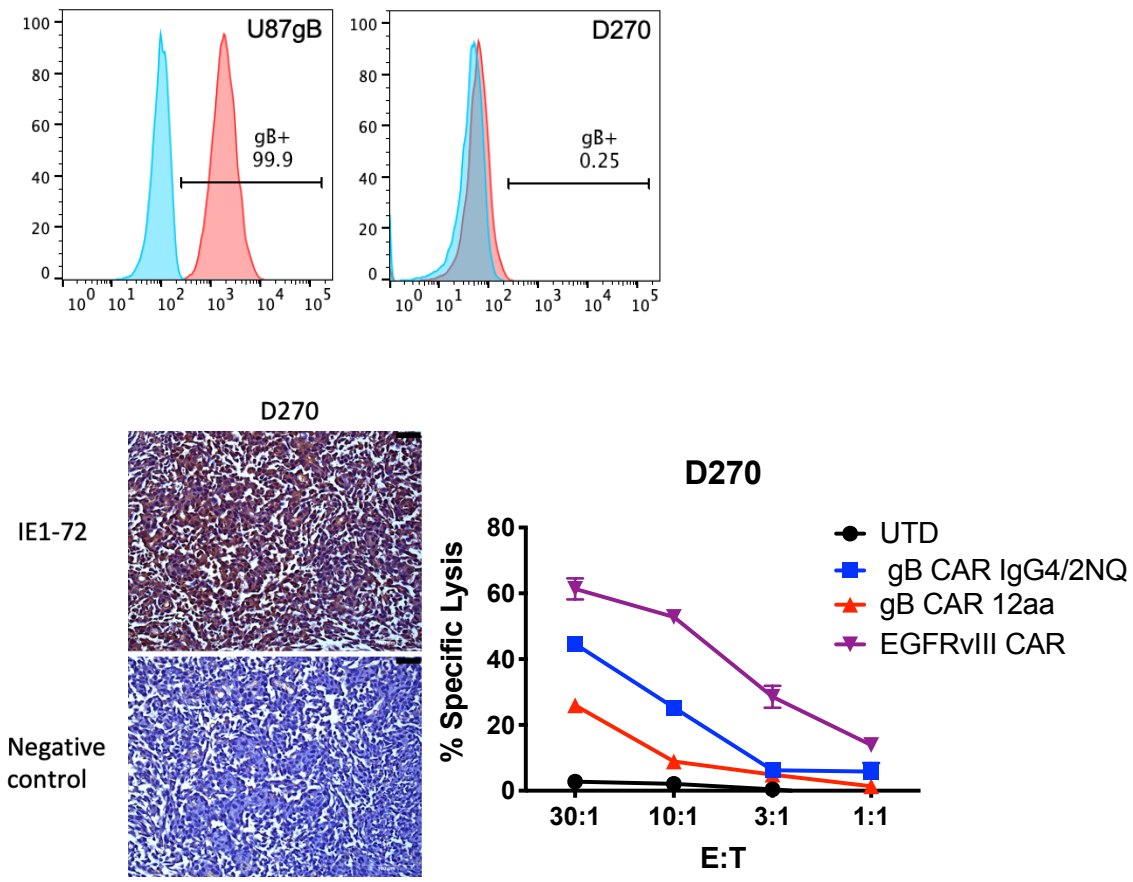

Figure 3.7. gB CAR T cells have anti-tumor activity against naturally-infected GBM explant D270. **A**. The human glioma xenograft explant cell line D270 was immunostained with anti-gB monoclonal and analyzed via flowcytometry. Human glioma cell line U87 transduced with glycoprotein B was used as positive control. **B.** Immunohistochemistry (IHC) for CMV antigen IE1-72 on brain tissue isolated from NSG mice after intracranial injection of D270 cells **C.** Cytolytic activity of gB CAR T cells against D270 in a 6hr chromium release assay. Shown are representative results from one donor. This assay was performed at least twice with 2 independent donors. Plots show means +/- SEM of triplicate wells.

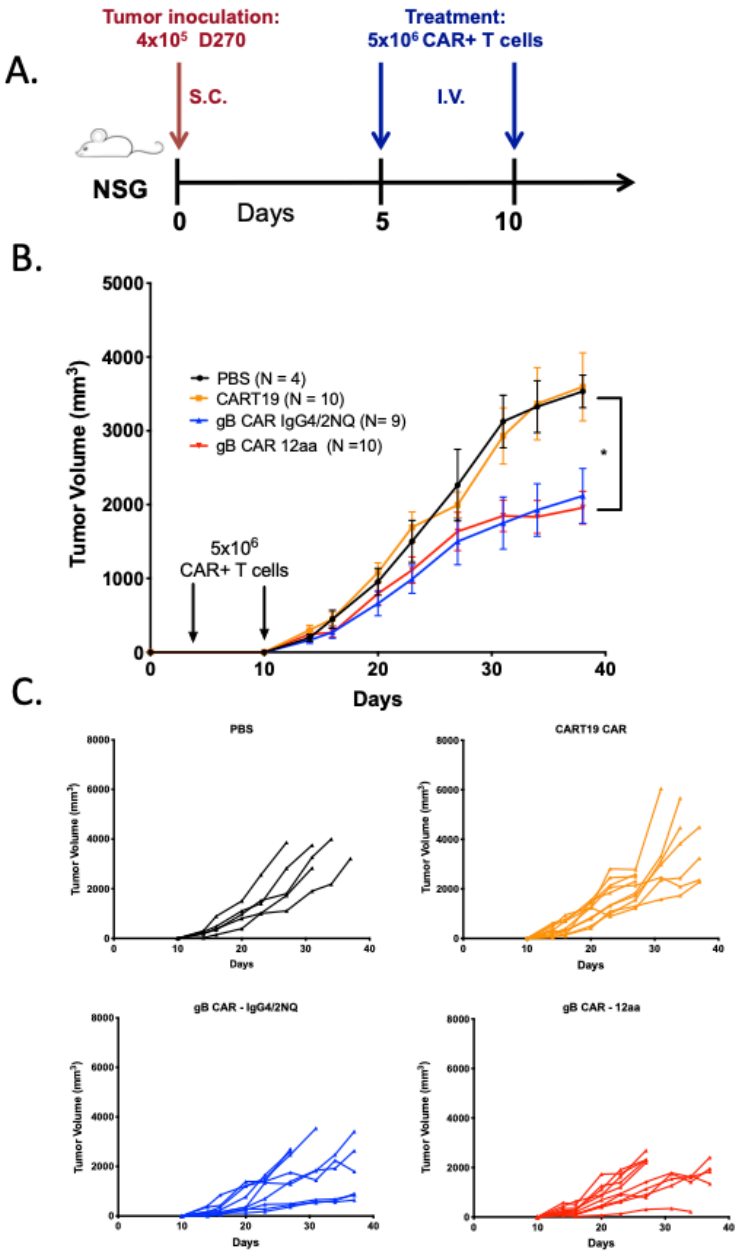

Figure 3.8. gB CAR T cells have anti-tumor activity against naturally-infected GBM explant D270 in vivo **A**. Schematic representation of a murine subcutaneous tumor model using NSG mice. NSG were inoculated subcutaneously with $4x10^5$ D270 cells (n=10 per group). Three days after tumor engraftment and again at day ten, each mouse was treated with $5x10^6$ CAR+ T cells per mouse injected intravenously. N=10 animals per group unless otherwise noted. **E.** Serial caliper measurements taken at regular intervals until the tumor reached the end point of 2cm in any dimension (LxWxH) or showed signs of graft versus host disease (GHVD). Each line represents the mean average of the group and standard error of the mean represented by the error bars. A two-way ANOVA was performed comparing experimental gB CAR T cells to the CD19 CAR T cell treated mice (* = $p<0.05$). **F.** Individual growth curves for each mouse per group until reaching the endpoint. Experiment was repeated three times with three independent T cell donors.

CHAPTER 4:

Discussion and Future Directions

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Discussion

We Can Rebuild It, We Have the Technology: Using gB CAR T Cells to Reconstitute HCMV Immunity

Inherent in the design of the gB CAR lies an alternative use in the context of HCMV reactivation in patients recovering from allogeneic hematopoietic stem cell transplantation (allo-HSCT) (Full et al., 2010; Moss & Rickinson, 2005; Proff et al., 2018). Adoptive cell transfer (ACT) of viral-specific T cells (VSTs) to control viral reactivation has been tested clinically with remarkable success (Harris et al., 2019; Papadopoulou et al., 2014; Riddell et al., 1992). These clinical trials demonstrate the feasibility of using ACT in controlling viral reactivation; however, with the exception of HIV, no clinical trials to our knowledge have reported the use of CAR T cells as the modality of treatment (Scholler et al., 2012). CAR T cells overcome a major limitation of *ex vivo* expanded viral-specific T cells by recognizing their cognate antigen in an HLA-independent fashion, thus allowing for the development of T cells that recognize shared and common target antigens on infected cells with universal application. Circumnavigating HLA-restriction allows for broad clinical application of virus-specific CAR T cells for patients with compromised or suppressed immune systems experiencing viral reactivation (Gandhi & Khanna, 2004; Jain et al., 2014).

There is promising clinical evidence from testing the efficacy and safety of VSTs to treat a wide range of viral infections to suggest that a CAR T cell-based approach would also work. Unlike cancer and HIV, where most clinical interventions work to delay disease progression, most of the infections treated with

VST therapies cannot be cleared by the endogenous, unmodified immune system. The problem lies in the inability of the patient to develop a new immune response or recall from memory effectors to common pathogens, such as HCMV, Epstein-Barr virus (EBV), and Adenovirus, after allo-HSCT (Moss & Rickinson, 2005). Ultimately, this becomes a clinical problem of accessibility. Rapid delivery of a VST or CAR T cell product may be able to suppress viral reactivation. Fortunately, there are a few ways to manufacture clinical products in a short amount of time (Bollard & Heslop, 2016). The safest approach would be to use a patient's own T cells to express the anti-viral CAR construct. A highly personalized autologous T cell product would be safer with regard to avoiding graft versus host disease (GVHD) than allogenic donor-derived VSTs. Autologous T cells can be lentivirally transduced for stable expression or electroporated with CAR mRNA for a rapid but transient expression of CAR (Foster et al., 2019; Maus et al., 2013). Transient expression of a TCR specific to hepatitis B virus (HBV) led to a drop in HBV viremia in a mouse model, suggesting that mRNA transfer of TCR or CAR could be a feasible approach to control viral infections (Kah et al., 2017). Autologous CAR T cells are likely to persist in patients recovering from allo-HSCT, such that a preemptive infusion of anti-viral CAR T cells could avert the complications associated with viral reactivation.

The second approach would be to use third-party donor T cells expressing anti-viral CARs. In this scenario, safety of the CAR T cell product is traded for convenience of utilizing a bank of "off-the-shelf" T cell products. Despite the safety precautions one must consider when using third-party donor T cells, there is

evidence to suggest that VST products can be safe (Leen et al., 2013). In a clinical trial using banked third-party VSTs, patients undergoing viral reactivation were infused with partial HLA-matched T cells. Recipients were given a T cell product if they shared at least one of out six potential class 1 HLA alleles. Surprisingly, no acute severe adverse events (SAEs) were reported after the VST products were administered to patients and only 2 patients out of 50 in the clinical trial developed *de novo* GHVD. Depending on the viral infection being treated, successful resolution from infection ranged from 66.7 % to 77.8% (Leen et al., 2013). A follow-up clinical study using a "mini" bank of HCMV-specific T cells derived from 8 donors reported a 100% response rate and no SAEs associated with administration of the T cell product (Tzannou et al., 2019). The safety and feasibility of using a highly enriched anti-viral T cell product as a platform for CAR expression makes VSTs a convenient source for donor-derived T cells due to their reported safety. Additionally, VSTs as a CAR expression platform can result in the development of CAR T cells with multiple specificities conferred via the endogenous TCR and CAR that could reconstitute anti-viral immunity simultaneously against multiple viruses. A final note on the matter, as gene-editing technology improves the ability to make universal CAR T cells, the potential to generate large stocks of banked anti-viral CAR T cell products from third party donors and administered "off-the-shelf" at medical centers may be realized (Bollard & Heslop, 2016; Qasim et al., 2017; Ren et al., 2017).

While the reported clinical efficacy of VSTs is promising as a modality to treat viral infections, the cost of the producing these cells can be prohibitory to its

implementation in the clinic and one must consider cost as a factor in whether a cell therapy is implemented to treat viral reactivation over conventional anti-viral drugs. One study focusing on the clinical and financial burden of HCMV reactivation after allo-HSCT found that 75.6% patients at risk for HCMV disease experienced HCMV reactivation at a median of 30 days post transplantation. Moreover, pre-emptive treatment of HCMV using antiviral drugs led to a mean increase of 13.9 days of additional inpatient hospitalization resulting in an estimated added cost ranging from $58,000 to $74,000 per patient (Jain et al., 2014). Similarly, a medical center in France found that the occurrence of one or more HCMV reactivation incidents could raise the total cost of allo-HSCT by as much as 25-30% (Robin et al., 2017). While treatment of HCMV reactivation with antiviral drugs may be convenient, it is not without complications and toxicities. Acute kidney damage and myelosuppression have been reported for some antiviral drugs commonly used to treat HCMV diseases (Jacobsen & Sifontis, 2010). The limited range of efficacy and continual dosing of these drugs complicate the clinical management of HCMV disease, which makes a compelling case for the use of HCMV CAR T cells or VSTs as a safer and effective alternative.

The high response rates reported using ACT of HCMV-specific T cells demonstrate that reconstitution of HCMV immunity is a biological possibility. It remains to be determined if gB CAR T cells will have comparable clinical activity. HLA-independent CAR T cell recognition of the target antigen reduces the associated costs of making HCMV-specific T cells for specific HLA alleles. Further cost reduction could be found in the relatively low dose required to reconstitute

HCMV immunity. One study used autologous polyclonal CD8+ and CD4+ HCMV-specific T cells with 15/18 patients responding to doses as low 1.2-116 x10^3cells/kg after allo-HSCT (Feuchtinger et al., 2010). Allogeneic CD8+ HCMV-specific T cells similarly eliminated HCMV infection in 8/9 patients with doses ranging from 1.2-33 x10^3 cells/kg (Cobbold et al., 2005). Independent of the T cell source, the low dose required to eliminate HCMV infection could translate into lower manufacturing costs. Additionally, as the cost of CAR T cell therapies is reduced by improvements to gene-editing, gene transfer technologies, and manufacturing, VST therapies can be implemented at medical centers as a safe and effective alternative to toxic antiviral drugs (Manufacturing reviewed in Levine, Miskin, Wonnacott, and Keir (2017)).

A Dream Antigen for a Sleeping Virus: Redirecting CAR T Cells to HCMV antigen US28

While the focus of this body of work is based on targeting gB, the breath of potential targets found within the HCMV genome should be taken into consideration (Gandhi & Khanna, 2004). Another HCMV antigen that can be targeted via CAR T cells is the HCMV-encoded chemokine receptor US28. US28 is a viral G protein-coupled receptor (vGPCR) that shares homology to human CCR1, CCR2, CX3CR1 and provides the virus the ability to scavenge host-derived signals with high promiscuity (Lee, Chung, & Lee, 2017). Studies into the function of US28 found that this vGCPR assists in the dissemination of HCMV by promoting cellular migration via chemotaxis (Streblow et al., 1999). Another report suggested

that US28 is a potential viral oncogene that confers an angiogenic phenotype and promotes tumor formation in cells stably expressing US28 (Maussang et al., 2006). Given these oncogenic properties, it is no surprise that US28 is expressed GBM (Dziurzynski et al., 2011; Soroceanu et al., 2011). Ectopic expression of US28 in primary GBM tumors leads to an invasive phenotype and increase of angiogenic activity via VEGF secretion (Soroceanu et al., 2011). Targeting approaches using a US28-specific nanobody resulted in a delay in tumor growth in a murine orthotopic tumor model of GBM (De Groof et al., 2019; Heukers et al., 2018). Unlike gB, expression of US28 has been reported in both lytic and latently infected cells (Cheung, Abendroth, Cunningham, & Slobedman, 2006; F. D. Goodrum, Jordan, High, & Shenk, 2002; Krishna et al., 2017). As a potential CAR target, US28 could pose toxicities by killing latently infected CD34+ bone-marrow progenitor cells and CD14+ monocytes that carry the HCMV (Sinclair & Sissons, 2006). Alternatively, depending on the distribution of HCMV among the CD34+ bone-marrow progenitor cells, therapeutic clearance of the HCMV reservoir could be achieved with manageable toxicities using US28-specific CAR T cells. US28 helps maintain HCMV latency, rendering latently infected cells susceptible to CAR T cell killing if expressed on the surface (Humby & O'Connor, 2015). Furthermore, loss of US28 expression results in the initiation of lytic infection and expression of a large subset of antigens, many of which can be detected by CD8+ T cells primed to HCMV antigens presented on class I HLAs (Krishna et al., 2016). The CD34+ compartment displays different subsets of cells that may not be amenable to HCMV replication and thus may be spared by US28-specific CAR T cells,

suggesting negligible US28-CAR mediated toxicities (F. Goodrum, Jordan, Terhune, High, & Shenk, 2004). gB and US28 represent two out of many potential CAR targets found within the large genome of HCMV. The expression patterns of these two antigens delineate potentially different levels of toxicities and therapeutic outcomes. This broadens the pool of potential targets that can be targeted with CAR T cells for GBM immunotherapy.

Getting to the Root of the Problem: Using gB CAR T Cells to Target Glioma Cancer Stem Cells

The emergence of the concept of the cancer stem cell (CSC) to explain the ability of a tumor remerge after treatment has gained traction in cancer research. CSCs constitute a small and rare fraction of the total tumor cell population but possess high proliferative capacity and self-renewal capability (Ma et al., 2018). Given these tumor re-initiating properties, therapeutically targeting CSCs would be beneficial to patient outcomes. Standard cancer therapies indiscriminately kill tumor cells and normal cells but do not result in total elimination of these tumor-initiating cells. In GBM, glioma CSCs (gCSCs) have been traditionally defined by CD133+ antigen expression; however, additional antigens, such as Notch1, SOX2, Oct4, and Nestin have also been used to define these cells. A series of reports have suggested that HCMV is enriched in the gCSC population or may directly induce a stem-like phenotype in infected glioma cells (Fiallos et al., 2014; Fornara et al., 2016; Soroceanu et al., 2015). These observations suggest that an HCMV-specific CAR T cell would selectively deplete tumor-reinitiating gCSCs and reduce

the likelihood of tumor reoccurrence. Other groups have reached similar conclusions on how immunological targeting of HCMV in GBM may lead to durable responses in patients with GBM (Rahman et al., 2019). It is worth noting that EGFRvIII expression has also been linked to gCSCs, but clinical use of an EGFRvIII CAR did not result in a halt of disease progression (Morgan et al., 2012; O'Rourke et al., 2017).

Tumor Associated Macrophages: GBM's Workhorse and HCMV's Trojan Horse

One of the interesting features of re-directing T cells to HCMV antigens is the possibility that gB CAR T cells could kill tumor-supporting macrophages. A large fraction of the GBM tumor mass can be attributed to myeloid cells, specifically tumor-associated macrophages (TAMs) and microglia (Graeber, Scheithauer, & Kreutzberg, 2002; Hussain et al., 2006). Similarly, when the immune infiltrate of GBM tumors was analyzed after treatment with neoadjuvant nivolumab, the myeloid lineage was found to be the most abundant non-tumor cell (Schalper et al., 2019). TAMs account for up to 30% of a GBM's total tumor mass and can subvert anti-tumor T cell responses via secretion of immune-inhibiting cytokines contributing to the immunosuppressive microenvironment of GBM (Wurdinger, Deumelandt, van der Vliet, Wesseling, & de Gruijl, 2014). While generally regarded as tumor supporting, TAMs can be re-educated to have antitumor activity. In a murine model of pancreatic ductal adenocarcinoma, the use of an anti-CD40 agonistic monoclonal antibody in combination with gemcitabine lead to tumor

regression by re-educating macrophages to develop M1 anti-tumor phenotype (Beatty et al., 2011). Systemic depletion of T cell subsets did not abrogate the treatment effect in tumor bearing mice, suggesting that macrophages possess tumoricidal activity independent of T cell help. The phenotypic plasticity displayed by TAMs can provide an antigen-independent mechanism of tumor killing that can complement CAR T cells armed with a narrowly defined specificity.

TAMs could also account for the presence of HCMV in many GBM tumors. While investigating the origin of GBM TAMs, one group found that most of TAMs originated from the bone marrow and are not tissue-resident microglia cells (Muller et al., 2017). This observation is interesting when paired with reports demonstrating that myeloid cells derived from HCMV-infected CD34+ progenitors are the only cells permissive for HCMV reactivation and replication in the periphery (Taylor-Wiedeman, Sissons, Borysiewicz, & Sinclair, 1991). In light of these reports, one can speculate that HCMV infected bone marrow-derived macrophages serve as a “Trojan horse” for HCMV to enter tumor sites and could explain the large tropism of this virus for many unrelated malignancies in addition to GBM. Once HCMV-infected monocytes enter the tumor microenvironment, the virus is allowed to spread primarily via a cell-to-cell process mediated in part by gB (Isaacson & Compton, 2009; Navarro et al., 1993). Experimental evidence to support the “Trojan horse” hypothesis comes from a report that identified TAMs and gCSCs as the cells within GBM tumors that harbor HCMV genomes. Moreover, when these GBM TAMs were characterized, they displayed an M2 immunosuppressive phenotype that was correlated with expression of HCMV viral

IL-10 (vIL-10) homologue (Dziurzynski et al., 2011). The immunosuppressive microenvironment of GBM provides a viral sanctuary for HCMV to replicate without fear of clearance from the immune system. The onco-modulatory contribution of HCMV proteins to GBM increases tumorigenicity, which results in an unlikely symbiotic relationship between HCMV and GBM (reviewed in (Dziurzynski et al., 2012)). As a therapeutic intervention, CAR T cells simultaneously targeting both gCSCs and TAMs could result in a lethal blow to the GBM support system; the roots and soil that permit GBM to persist could be rendered so inhospitable after anti-gB CAR T cell treatment that tumor reoccurrence may be delayed or averted entirely. However, if TAMs cannot be depleted, they could be skewed to an anti-tumor phenotype. As demonstrated by (Pyonteck et al., 2013), pharmacological inhibition of the colony stimulating factor-1 receptor (CSF1-R) in a murine glioma model resulted in a reduction of TAMs with M2-like phenotype and delayed GBM tumor growth. This would suggest that a similar depletion or modulation of glioma-associated macrophages would confer a survival benefit to patients with GBM. Lastly, GBM tumors undergo tumor evolution from the initial diagnosis to recurrence. Nearly half of GBMs that have been molecularly subtyped as one subtype often morph to another and this evolution results in an altered immune-infiltrate (Q. Wang et al., 2017). Particularly, loss of the NF1 tumor suppressor gene in the mesenchymal subtype of GBM results in a marked increase of M2 macrophages that render GBM tumors radio-resistant.

These studies underscore the importance of TAMs as immunotherapy targets. As mentioned before, macrophages possess T cell-independent anti-

tumor activity (Beatty et al., 2011; Pyonteck et al., 2013). Promoting anti-tumor macrophage phenotypes or depletion of M2 macrophages would provide a general mechanism of anti-tumor activity and go beyond the limited scope of targets that CAR T cells can recognize on the tumor.

Modulation of HCMV Antigen Expression via Epigenetic Modulation of HCMV Latency

Viral latency allows virus to persist in hosts with minimal detection by the immune system. Because viral replication is not occurring, even the best anti-viral therapies fail to prevent eradication of the viral reservoir. This is perhaps best exemplified in the treatment of HIV with ART and the daily dosage required to keep the virus latent. However, if a virus could be driven out of latency, then therapeutic viral clearance can be achieved. This approach is being evaluated to clear HIV out of latency in combination with HIV-specific T cells, a “kick-and-kill;” strategy (S. H. Huang et al., 2018). One approach to drive a virus out of latency is through the use of HDAC inhibitors (HDACi). As it relates to HCMV, one report demonstrated that a class II HDACi was sufficient to trigger transient lytic infection and make cells harboring latent virus susceptible to killing by HCMV-specific T cells (Krishna et al., 2016). As a parallel, anti-gB CAR T cells could demonstrate similar activity on HDACi-treated tumor cells to drive expression of gB. The molecular events that lead to lytic infection are tied to expression of the HCMV immediate early 1 and 2 (IE1 and IE2) gene products. The major immediate early promoter (MIEP) controls expression of IE1 and IE2 and during latency is kept in a repressive chromatin

state by HDAC3 activity (Murphy, Fischle, Verdin, & Sinclair, 2002). Specific inhibition of HDAC3 could therefore jumpstart HCMV lytic gene expression, including expression of gB.

If HDACi approaches do not work, targeting the regulators of HCMV latently could be an alternative approach. HCMV US28 expression on latently infected cells has been reported to help maintain MIEP in a repressive state. This activity has led to the development of therapies that seek to block the activity of US28 to eradicate the HCMV reservoir (Elder & Sinclair, 2019). A US28-specific nanobody can stop the constitutive activity of US28 by blocking ligand binding and lead to lytic gene expression (Heukers et al., 2018). As previously discussed, surface expression of US28 makes this HCMV antigen a potential CAR target. The ability of CAR T cells to home to sites of antigen expression could lead to direct eradication of the HCMV latent reservoir. Alternatively, the ability of T cells to trogocytose and deplete US28 on latently infected cells could also trigger lytic gene expression (Hamieh et al., 2019). CAR T cells simultaneously targeting both US28 and gB would work on distinct phases of the HCMV life cycle. Additionally, blocking of US28 activity via nanobody or CAR could sensitize HCMV infected cells to killing via gB expression or other intracellular antigens such pp65 or IE1 that T cells are primed to detect.

gB CAR T cell Associated Toxicities: Known Knowns and Known Unknowns

Genetically re-directing T cells to new specificities can be fraught with danger, resulting in immune destruction of non-malignant tissues that express the

target antigen. HCMV and other viral-associated tumor antigens may offer a greater safety profile than traditional TAAs derived from self-antigens as viral antigens are intrinsically foreign to the patient's immune system. One can draw inferences from the natural immune response to HCMV and how this could predict safety of anti-gB CAR T cells in the clinic. Acute infection with HCMV leads to robust cellular and humoral immune response in an infected host, typically with no development of overt symptoms of infection. Resolution of HCMV infection leads to a latent infection with no associated autoimmune disease. The human host can develop a robust immune response to HCMV and its gene products, as they are foreign antigens that are readily recognized by the immune system, thus breaking immunological tolerance. For this reason, humoral and cellular responses to HCMV can be readily detected in patients. This feature is reflected in the construction of our gB CAR. The scFv used to generate our gB CAR was derived from a healthy human donor that experienced CMV infection and developed antibodies to gB (Ohlin et al., 1993).

The safety of gB CAR T cells draws inferences based on the typical mechanisms of how a T cell or an antibody-producing B cell recognizes antigen. CAR T cells sense antigen in an HLA-independent manner and this could be a source of unexpected toxicities. As mentioned earlier, trastuzumab (Her2 mAb) does not cause lung toxicities; however, when the same Her2 scFv was used in a CAR construct, the first and only patient treated experienced lethal acute lung toxicities (Morgan et al., 2010). Following this logic, similar toxicities can occur due to the atypical recognition of gB antigen on normal tissue by anti-gB CAR T cells

that would be undetectable if the anti-gB targeting occurred via an antibody-dependent mechanism. Experimentally, the increased sensitivity of CAR T cells to low levels of antigen has been demonstrated when comparing BiTE molecules to CAR T cells bearing the same scFv (Stone et al., 2012). The degree of sensitivity CAR T cells have towards their cognate antigens has been the subject of speculation. An initial determination of the sufficient amount of molecules needed to trigger cytolytic function of an anti-CD20 CAR T cell was estimated to be approximately 200 CD20 molecules on the surface of the target cell (K. Watanabe et al., 2015). Refinement in tools used to measure antigen density has shown thatin some cases with optimized CARs, less than 100 molecules of CD19 or CD20 are sufficient to trigger T cell lysis of a tumor cell (Nerreter et al., 2019). The exceedingly low levels of antigen needed for CAR T cells to kill a target cell may uncover toxicities on normal tissues, which may lead to pathology not typically associated with HCMV gB-specific antibodies.

Another factor is the possibility that anti-gB CAR T cells could cross react with self-antigens that have similar epitopes as the CAR or other herpes virus family gB antigens that share a highly conserved protein structure (Heldwein et al., 2006). While these safety concerns highlight unknown toxicities, known toxicities can occur in cells that harbor the HCMV reservoir. CD34+ myeloid progenitor cell are a well-established reservoir of HCMV (Sinclair & Sissons, 2006). Reports profiling expression of HCMV genes in latently-infected CD34+ myeloid progenitors do not report gB/*UL55* gene expression, as it is considered a marker of early-to-late productive lytic infection (Cheung et al., 2006; F. D. Goodrum et al.,

2002; Stern & Slobedman, 2008). The shift from HCMV latency to reactivation occurs in monocytes undergoing differentiation at which point expression of gB can be detected (Soderberg-Naucler et al., 2001). Even if gB CAR T cells were to eliminate all HCMV harboring monocytes, this would not result in a monocyte deficiency. One estimation found that the frequency of peripherally latently-infected mononuclear cells from 12 patient-derived samples was approximately 0.004% to 0.01% of the total cell population (Slobedman & Mocarski, 1999). This exceedingly rare population of HCMV-infected cells in an immune-competent host would predict negligible “off-tumor, on-target” toxicities.

CAR T Cells Redirected to HCMV May Have Superior Anti-Tumor Activity than Ex-vivo Expanded HCMV-specific T Cells

The success of ACT therapies can depend on the quality of the T cell product. One of the features distinguishing CD19BBz CAR T cells from those bearing CD28 costimulation is the enrichment of the central memory (CM) phenotype (Kawalekar et al., 2016). Delving deeper, one group investigated the anti-tumor properties of different T cell memory subsets and established that a less differentiated T cell has a higher proliferative capacity and this correlated with persistent anti-tumor activity (Gattinoni et al., 2011). As it relates to HCMV-specific T cells, one must consider the memory state of the antigen-specific pool, especially if they are using in ACT therapies with *ex vivo* expanded HCMV-specific T cells. Chronic infection of HCMV leads to effector memory differentiation of T cells due to prolonged antigen exposure (Pardieck, Beyrend, Redeker, & Arens, 2018; Vieira

Braga, Hertoghs, van Lier, & van Gisbergen, 2015). Clinical trials using ACT of HCMV-specific T cells to treat GBM may have resulted in poor clinical outcomes perhaps due, in part, to the differentiation state of the T cell product (Ahmed et al., 2017; Ghazi et al., 2012; Schuessler, Smith, et al., 2014). The series of work using dendritic cell vaccines pulsed with pp65 have been promising in that they generate poly-functional T cells (Reap et al., 2018). An alternative approach to overcome this inherent barrier of using endogenous HCMV-specific T cells is to re-direct T cells using a CAR. Differentiation data from the gB CAR T cells demonstrate that anti-HCMV CAR T cells bear a central memory phenotype. A report investigating tumor-infiltrating T cells found HCMV-specific T cells present in GBM tumors; however, these cells displayed a tolerized and exhausted phenotype (Bahador et al., 2017). Moreover, in a clinical trial that used *ex vivo* expanded CMV-specific T cells to treat GBM, TILs were analyzed from a patient that developed progressive disease 4 months after treatment and the HCMV-specific T cells displayed a high degree of exhaustion makers. Nearly half of the CMV-specific T cells were unable to respond to stimulation (Schuessler, Smith, et al., 2014). These studies would suggest that endogenous T cells primed to HCMV via infection recognize HCMV antigens in GBM tumors but do not impact tumor growth due to an exhausted phenotype. CAR T cells re-directed to HCMV may fair a bit better in tumor microenvironments due to the contribution of the CAR co-stimulatory domain, resulting a less differentiated state than *ex vivo* expanded HCMV-specific T cells.

Follow Up studies

This body of work has yielded a new CAR target for the treatment of GBM, yet some questions remain that require more thorough investigation. The remaining section will outline experiments that will help improve the clinical implementation of anti-gB CAR T cells against GBM.

Experimental Determination of gB CAR T Cell Safety and Toxicities

The section on safety considerations outlines the natural history and immune response to CMV as predictors of the safety profile of gB CAR T cells. Experimentally, the safety of profile of gB CARs can be tested using a panel of primary human cells representing various tissues and organs. This panel has been previously been used to determine the on-target, off-tumor toxicities of an affinity-tuned CAR T cells re-directed to either EGFR or HER2 (Liu et al., 2015). Particular emphasize could be placed on cells deemed to be reservoirs of HCMV, such as CD34+ myeloid progenitor cells/HPSC (Sinclair & Sissons, 2006). A useful model to guide the development of our toxicity models is a report on CAR T cells re-directed to CD30 for Hodgkin's and non-Hodgkin's lymphomas. Due to potential “on-target, off tumor” complications, this study focused on the potential for toxicities in CD30+ HPSCs. Despite CD30 expression, HSPCs were not killed by CD30 CAR T cell due, in part, to lower levels of target expression that was insufficient to trigger CAR T cell killing. Additional mechanisms of resistance stems from HSPCs expressing SP6/PI-9 serine proteases that inactivate granzyme B-mediated killing (A. A. Hombach et al., 2016). The resilience of HPSCs to CD30 CAR T cell-

mediated killing may bode well for the safety of gB CAR T cells. An experiment that will directly address this question is *ex vivo* co-culture of CD34+ HPSCs from HCMV+ donors with gB CAR T cells. The degree and magnitude of toxicities against HPSCs could be inferred from this experiment. Additionally, the normal cell panel reported by Liu *et al.* could be used to detect killing of normal tissues that express antigens that may cross-react with the epitope of ITC52 scFv-based gB CAR (Liu et al., 2015). Due to the broad tropism of HCMV, it is possible that gB CAR T cells may kill HCMV-infected cells of various tissue origins; however, clinical presentation of these toxicities may not differ from that of endogenous T cell mediated killing of infected cells during HCMV acute infection.

Improving the Sensitivity of gB CAR T Cells to Low Levels of Antigen Expression

Low levels of gB antigen expression on GBM tumors could limit the efficacy of gB CAR T cells. Despite optimization of the hinge region to improve cytokine production *in vitro*, these modifications may not be enough to trigger CAR T cell effector function if gB antigen on primary GBM is found below an activation threshold (A. A. Hombach et al., 2016; K. Watanabe et al., 2015). Fine-tuning the signal strength mediated by CAR signaling domains may be an alternative approach to improving gB CAR T cell function. Sensitization of a CAR T cell to target antigen can be achieved by the incorporation of additional ITAM domains into the CD3ζ chain domain of first generation CD19 CAR construct. Typical CAR constructs include 3 ITAM domains; however, with the addition of more ITAMs,

CD19 CAR T cells were able to recognize $CD19^{low}$-expressing targets (J. R. James, 2018). This modification could be introduced into the design of the second generation gB CAR constructs to improve the sensitivity to gB antigen. The sensitivity of gB CAR T cells with additional ITAMs can be tested by dosing gB antigen into target cells via mRNA electroporation. Both cytotoxicity assays and cytokine release assays can be used as readouts to determine if additional ITAMs improve the sensitivity of gB CAR T cells. A variation of this experiment was reported for HER2 affinity-tuned CAR T cells. Dosing of increasingly low amounts of HER2 mRNA into target cells was sufficient to trigger CAR T cell degranulation in a HER2-specific CAR T cells. The addition of ITAMs or a different signaling domain can be compared using the experiments outlined in (Liu et al., 2015).

Studies comparing the anti-tumor function of CAR T cells that incorporate either the CD28 or CD137 (4-1BB) co-stimulatory domain rely upon models where target antigen expression is well above the threshold needed to trigger CAR T cell function. In tumor models where antigen is limiting due to low expression, incorporation of a CD28 co-stimulatory domain could potentially improve sensitivity and anti-tumor function of CAR T cells due to stronger signal provided by CD28 and less frequent engagement with target antigen (Salter et al., 2018; Walker et al., 2017). CAR T cells can diminish the level of target antigen on a tumor cell via the process of trogocytosis. This mechanism of lowering antigen density was true for both CD19 CAR using either a CD28 or a 4-1BB co-stimulatory domain. However, it was only in the cohort of tumor bearing mice treated with CD1928z CAR T cells that clearance of $CD19^{low}$ antigen tumors was observed (Hamieh et

al., 2019). These modifications underscore the importance of fine-tuning of signal strength for the particular antigen being targeted, as many of the reports demonstrating the superior anti-tumor activity of CAR T cells bearing the 4-1BB co-stimulatory domain are based on tumor models where antigens were expressed at normal levels.

Broadening the Range of Clinical Strains of CMV Covered by gB CAR T Cells

Immunological pressure exerted upon any epitope could result in the development of escape variants. This problem is particularly pronounced when targeting viral antigens. The scFv of ITC52 used to develop this gB CAR recognizes the AD-1 epitope of gB, which is highly conserved among HCMV strains. Mutations introduced into the AD-1 domain resulted in loss of infectious virus particles produced, suggesting that viral fitness is compromised if this domain undergoes mutations (Britt, Jarvis, Drummond, & Mach, 2005). Factoring the mutational constraints that gB AD-1 can handle, the likelihood of encountering escape variants is greatly diminished for gB CAR T cells targeting this epitope. However, additional epitopes within gB have been reported along with new human donor derived scFvs that recognize additional antigenic domains (Ohlin & Soderberg-Naucler, 2015). I have generated a new set of gB CAR constructs based on the SM5-1 scFv that recognizes the AD-5 domain of gB (Potzsch et al., 2011). Other groups have published abstracts using the SM5-1 scFv in their gB CAR construct, indicating that this scFv can be used to re-direct CAR to gB (Olbrich et al., 2020). A tandem-CAR design (i.e. tan-CAR) approach could be used to

target gB with a two-fold benefit. The use of two scFvs that recognize gB will reduce the likelihood of a HCMV strain developing two simultaneous escape mutations (Grada et al., 2013). The second benefit would be an improvement in the molecular avidity of a gB CAR molecule for gB by providing two points of contact and, as a result, could sensitize gB CAR T cells to low levels of gB antigen presumed to be found on GBM tumors.

Of Mice and Non-Human Primates: Animal Models to Test Control of HCMV Reactivation by gB CAR T Cells

Many of the experiments designed to test the anti-tumor function of gB CAR T cells can be translated to the potential use of gB CARs in the context of HCMV reactivation. HCMV is a species-specific virus and cannot infect non-human cells, leaving no viable *in vivo* infection model for HCMV. However, the recent development of a humanized bone liver thymus (BLT-NSG) mouse model can now be used to interrogate the *in vivo* function gB CAR T cells (Crawford et al., 2017). NSG mice engrafted with human donor bone marrow, liver, and thymus develop a normal immune system capable of mounting an immune response comparable to that seen in human hosts. The benefit of an *in vivo* infection model would be two-fold. First, one could study the ability of gB CAR T cells to control HCMV in the context of chronic infection and the persistence of gB CAR T cells. Secondly, toxicities associated with CD34+ progenitor cells could be evaluated in a dynamic model of HCMV latency if the BLT model reproduces human HCMV latency. Simpler models of HCMV reaction have also been reported. NSG mice engrafted

with only human CD34+ progenitor cells give rise to monocytes capable of carrying latent virus (Crawford et al., 2019). The degree of anti-viral activity gB CAR T cells have against HCMV infection can be measured by the genomic copies of the virus found in the mouse liver and spleen. A reduction in HCMV genome copies/ µg of DNA in these organs could signify anti-viral gB CAR T cell activity (Crawford et al., 2019).

Large vertebrate animal models could be more predictive of clinical outcomes in humans. Rhesus macaques have a species-specific CMV (RhCMV) that closely mimics the biology of HCMV (Powers & Fruh, 2008). The similarity between these two viruses also extends to the high degree of amino acids conservation of their respect gB antigen (Kravitz, Sciabica, Cho, Luciw, & Barry, 1997). Of note is a report demonstrating antibodies generated against the human gB can cross react with RhCMV gB (Kropff & Mach, 1997). One of the mAbs tested for cross reactivity is the mouse mAb 27-287 that can detect both HCMV and RhCMV gB. mAb 27-287 recognizes the AD-1 region of gB and shares a similar epitope with the ITC52 human mAb used to generate my gB CAR. This would suggest that the ITC52-based gB CAR could also cross react with the RhCMV gB and be used in rhesus macaque models of CMV reactivation. Gene transfer of CAR constructs into rhesus macaque T cells has been previously reported, allowing for direct testing of the anti-viral activity of gB CAR T cells (Taraseviciute et al., 2018). Experimental determination of cross-reactivity could be tested by ectopic expression of RhCMV gB in human target cells and measuring cytotoxic and cytokine activity of gB CAR T cells.

Future Directions

The primary goal of this study was to test the hypothesis that human cytomegalovirus is a viable target for CAR T cells in GBM. The high prevalence of the virus in GBM tumor samples and high immunogenicity make it an attractive tumor target. Moreover, the immune systems' ability to recognize and mount an immune response to many of the gene products found in the HCMV genome provide an opportunity to generate epitope spreading. Induction of epitope spreading is a highly desirable feature of cancer immunotherapies and may help to overcome the antigen heterogeneity found within tumors.

The gB CAR detailed in this thesis serves as a prototype for further design improvements. HCMV expresses other surface antigens that can also be detected with CAR T cells and targeted with or instead of gB. Perhaps the most promising feature of a gB CAR is the many indications this CAR may treat. In addition to GBM, HCMV has been detected in breast cancer, colorectal cancer, and low-grade gliomas. However, the most direct application of a gB CAR T cells would be in the treatment HCMV reactivation in patients that have received allogeneic stem cell transplant.

Closing Remarks

"Illness is the night side of life, a more onerous citizenship. Everyone who is born holds dual citizenship, in the kingdom of the well and in the kingdom of the sick. Although we all prefer to use the good passport, sooner or later each of us is obliged, at least for a spell, to identify ourselves as citizens of that other place."

— Susan Sontag, Illness as a Metaphor

As Susan Sontag reflected on the duality of wellness in her essay Illness as a Metaphor, she highlighted the human attributes we attach to diseases due to lack of understanding of a disease (Sontag, 1979). Cancer, perhaps the most mysterious illness at the time aptly fit the metaphor of the dual passport Sontag described. At the time of her writing, clinicians and cancer biologists were only starting to understand the molecular mechanisms that drive cancer. The work of Bishop and Varmus in the 1970s began to illuminate the oncogenic origins of cancer. Their seminal work lead to the discovery that the very genes that can cause cancer are sewn into our genome (Stehelin et al., 1976). Given enough time, one of the cells that constitutes our being could undergo cellular transformation and we would "identify ourselves as citizens of that other place" (Sontag, 1979). But genome-derived oncogenes do not tell the whole story, as tumor-associated viruses in their quest to replicate will also turn on or inactivate gene products that can initiate the process of tumorigenesis (Krump & You, 2018). In the decade that followed the works of Bishop and Varmus, reports of a handful of patients presenting with a rare malignancy and a dysfunctional immune system marked some of the first cases of HIV/AIDS. As it later became clear, the rarely seen Kaposi's sarcoma was allowed to manifest when HIV had ravaged the patient's

immune system. The culprit virus, KSHV, unchecked by an active immune system was allowed to transform infected cells unabated. KHSV is just one of a handful of tumor-associated viruses that account for up to 20% of all human cancers (Krump & You, 2018). The high risk of viral-associated malignancies in patients with HIV/AIDS underscores the importance of the immune system for keeping some cancers at bay (Shiels & Engels, 2017). In the later end of the decade, the first iteration of what would become a CAR was first published (Gross, Waks, & Eshhar, 1989). Advances in understanding T cell biology led to improvements upon the CAR design, which eventually led to the approval of the first gene-therapy in the United States for CAR T cells targeting CD19 in ALL (Maude et al., 2014). A historical perspective is warranted to reflect on where cancer immunotherapy has been and where it is going. Gene editing technologies, such as CRISPR, have made genetic manipulation of cells a routine laboratory procedure. First-in-human trials using CRISPR-edited T cells demonstrated that gene-edited T cells can be safely administered to patients (Stadtmauer et al., 2019). New reports that provide mechanistic insights into new ways to augment the anti-tumor function of T cells are published every day. However, despite the optimistic outlook of CAR T cell technology in treating human diseases, it is constrained by a fundamental problem: how to distinguish what is safe to target on a cancer cell and what is not?

To the cancer immunotherapist looking for a tumor-associated antigen to target, viral antigens represent a treasure trove of potential targets. The association of HCMV with GBM represents an unlikely solution to the immunotherapist seeking to find an antigen unique to GBM and not on normal cells

(C. S. Cobbs et al., 2002). In the 15 years that have passed since the initial publication that defined the standard of care for GBM, no other clinical intervention has made a meaningful difference in the prognosis of patients with GBM (Stupp et al., 2005). This body of work is based on the controversial presence of HCMV in GBM. Methodological approaches undertaken by different labs to detect HCMV in GBM tumors have yielded divergent results. The robustness by which other tumor causing viruses can be found in tumors cannot be said for HCMV and GBM (Dziurzynski et al., 2011). Nevertheless, the foreign and highly immunogenic nature of the virus to the human immune system that made it the interest of different research groups hoping to leverage the presence of HCMV in GBM as a way to target GBM. And it is only now with the advent of CAR technology that a viral antigen can be a shared tumor antigen. CAR T cells bypass HLA-mediated presentation of antigens that made a viral epitope unique to that patient's HLA.

Robert Gallo's recounting of his search and discovery of the first human retrovirus to cause cancer parallels the story of HCMV and GBM. Prior to the discovery of human T-lymphotropic virus (HTLV-1), the search for human tumor retroviruses proved to be a fruitless endeavor, fraught with many false positives. The discovery of HTLV-1 was made possible by advances in technology that were able to sensitively probe for retroviral mRNA and developments in methods to culture human T cells (Gallo, 2005). Similarly, the confluence of fortuitous discoveries may be required to settle the story of HCMV and GBM. The parable of a group of blind men trying to describe an elephant comes to mind. Multidisciplinary approaches have yielded different insights in the biology of HCMV

in GBM but the whole image remains elusive. Is this association real, and if so, would it have a clinical impact as a target for patients with GBM? This body of work adds immunological evidence to the association of HCMV and GBM. Pursuing HCMV as a tumor antigen is a risky undertaking but, if successful, HCMV's gB can now be added to the arsenal of antigens that can be safely targeted on a tumor. GBM's high antigenic heterogeneity poses the greatest challenge for the application of CAR T cell therapy (Brown et al., 2016; O'Rourke et al., 2017). A malignant Hydra of Greek mythology: for each tumor cell clone eliminated by a CAR T cell, another clone impervious to CAR T cell recognition will take its place. As intractable as GBM may seem be to clinical intervention, CAR T cells pose a formidable challenge. Dynamic, expanding, and adapting to an ever-shifting foe, the "living drugs" that CAR T cells constitute may eventually fare better than other forms of cancer treatment. Each refinement upon a CAR's design and augmentation in the antitumor activity of a T cell chips away at the edifice that tumors represent.

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